

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> :		A1	(11) International Publication Number: <b>WO 98/51323</b>
A61K 38/00, 31/04, 31/10, 31/16, C07K 14/00			(43) International Publication Date: 19 November 1998 (19.11.98)
(21) International Application Number: PCT/US98/09691		(74) Agents: ASHTON, Nina, M. et al.; Cooley Godward LLP, 5 Palo Alto Square, 3000 El Camino Real, Palo Alto, CA 94306-2155 (US).	
(22) International Filing Date: 12 May 1998 (12.05.98)			
(30) Priority Data: 60/046,394 13 May 1997 (13.05.97) US		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/046,394 (CON) Filed on 13 May 1997 (13.05.97)			
(71) Applicant (for all designated States except US): THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 12th floor, 1111 Franklin Street, Oakland, CA 94607-5200 (US).		Published <i>With international search report.</i>	
(72) Inventors; and (75) Inventors/Applicants (for US only): WEINER, Richard, I. [US/US]; 230 Sunset Way, Muir Beach, CA 94965 (US). MARTIAL, Joseph, A. [BE/BE]; 13, rue Vignoble, B-4130 Esneux (BE). STRUMAN, Ingrid [BE/BE]; 64, rue de Huy, B-4317 Faimes (BE). TAYLOR, Robert [US/US]; 118 Granville Way, San Francisco, CA 94127 (US).			
(54) Title: NOVEL ANTIANGIOGENIC PEPTIDE AGENTS AND THEIR THERAPEUTIC AND DIAGNOSTIC USE			
(57) Abstract			
<p>The current invention concerns novel antiangiogenic peptides which correspond to about 10 to about 150 consecutive amino acids of N-terminal sequence of human growth hormone, human placental lactogen, human growth hormone variant hGH-V, and prolactin, and their use in inhibiting angiogenesis and in the diagnosis of diseases of human pregnancy involving abnormalities of placental vascularization.</p>			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## NOVEL ANTIANGIOGENIC PEPTIDE AGENTS AND THEIR THERAPEUTIC AND DIAGNOSTIC USE

This application claims the benefit of the priority date of and is a continuation in part of U.S. 60/046,394, filed May 13, 1998, which is incorporated herein by reference in its entirety for all purposes.

5

### BACKGROUND OF THE INVENTION

A growing number of serious, debilitating and often fatal diseases are associated with angiogenesis. These diseases are cumulatively called angiogenic diseases. Under normal physiological conditions, angiogenesis in mammals is 10 endogenously controlled throughout the lifetime and neovascularization rarely occurs except during embryonic development, the reproductive cycle, and wound healing.

Examples of the pathological conditions leading to development of angiogenic diseases are, among others, 15 arthritis, rheumatoid arthritis, atherosclerotic plaques, corneal graft neovascularization, wound healing, hypertrophic or keloid scars, proliferative retinopathy, diabetic retinopathy, macular degeneration, granulations, neovascular glaucoma and uveitis.

20 Additionally, rapid and excessive angiogenesis accompanies the growth of the placenta and solid tumors. Many tumors seem to produce factors which increase cell division of vascular endothelial cells and stimulate the migration and organization of endothelial cells into vessels resulting in neovascularization. In addition, factors which inhibit 25 angiogenesis may be turned off. Activation of angiogenesis, therefore, appears to be an essential stage in tumor progression. Various mammalian endogenous molecules have been identified as angiogenic factors stimulating angiogenesis 30 directly or indirectly. These stimulators of angiogenesis

include basic fibroblast growth factor (bFGF), vascular endothelial cell growth factor (VEGF), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and angiogenin.

The search for neovascularization inhibitors has been recently vigorously pursued. Currently, several antiangiogenic factors including thrombospondin, platelet factor-4, fumagillin, thalidomide angiostatin and endostatin are being studied. Several of these are in early clinical trials, e.g., fumagillin and thalidomide.

Since there is no effective treatment available and since angiogenic diseases present a serious medical problem there is an ongoing need for new and more efficient antiangiogenic agents.

Human hormones, such as growth hormone (hGH), prolactin (hPRL), placental lactogen (hPL) or growth hormone variant (hGH-V) are homologous protein hormones which are potent endogenous chemical substances asserting specific biological activities on their respective target organs. The biological activities of these hormones are not the same and they differ depending on the hormone and/or the hormone target organ. For example, growth hormone, which is a protein of the anterior lobe of the pituitary gland, promotes and regulates body growth and morphogenesis, fat mobilization and inhibitions of glucose utilization (*Trends Endocrinol.* Metab., 3:117(1992)).

Prolactin is a known hormone produced by the pituitary gland in all mammals. The normal biological function and activity of prolactin in mammals include regulation of reproduction, osmoregulation, the stimulation of milk production by the mammary gland, the modulation of steroidogenesis in the gonads, the stimulation of maternal behavior, and the modulation of immune function (*Life Sci.*, 57:1 (1995)).

The human placenta expresses two proteins with significant structural homology to human growth hormone, namely human placental lactogen and a variant of human growth hormone, hGH-V, differing by 13 amino acid substitutions (*Hormonal Proteins Peptides*, 4:61 (1977)).

Human placental lactogen is somatotropic in fetal tissues and aids in stimulating mammary cell proliferation- (*Endocrin. Rev.*, 12:316 (1991)). Human growth hormone variant rather than pituitary growth hormone regulates maternal metabolism during the second half of the pregnancy (*Endocrinology*, 133:1292 (1993)). As described in *Endocrinology*, 121:2055 (1987) rodent placentas express and secrete several proteins such as proliferin and proliferin-related peptide possessing biological actions similar to prolactin rather than growth hormone.

The human placenta serves as the major respiratory, nutritional and endocrine organ throughout fetal life and is critical to the survival and healthy development of the fetus. The placenta provides an intimate interface between the maternal and fetal blood supplies. Pathological impairment of placental invasion and poor vascular development has been associated with both fetal and maternal complications.

Intrauterine fetal growth restriction (IUGR) is a condition that affects approximately 500,000 pregnancies annually in the United States. As a result of poor placental blood supply, and decreased fetal oxygenation and nutrition, this condition results in small-for-dates infants. These infants have a neonatal mortality rate 6-10 times higher than that of normal infants and are at risk for pulmonary and neurological problems at birth.

Preeclampsia, a condition that affects approximately 250,000 American pregnancies each year, puts mothers and their fetuses at high risk. Maternal hypertension, renal failure, hepatic failure, coagulopathy, cerebral edema seizures and stroke are the potential consequences of this syndrome. Fetal morbidity is 5 times higher than normal in preeclampsia.

Pathological conditions involving excessive trophoblastic invasion, such as placenta accreta and gestational trophoblastic tumors, can cause devastating maternal complications including hemorrhage and metastatic neoplasia.

All the above placental conditions associated with dysregulation of the vasculature of the developing placenta

can have profound maternal and child health consequences.

Thus, there is a need for diagnosing and treating vascular abnormalities of the placenta. These needs and others are addressed by the instant invention.

5

#### SUMMARY OF THE INVENTION

One aspect of the invention is an anti-angiogenic peptide substantially identical to about 10 to about 150 consecutive amino acids selected from the N-terminal end of human placental lactogen, human growth hormone, growth hormone variant hGH-V, or human prolactin, wherein the peptide

(I) inhibits capillary endothelial cell proliferation and organization;

(ii) inhibits angiogenesis in chick chorioallantoic membrane; and

(iii) and binds to at least one specific receptor which does not bind an intact full length growth hormone, placental lactogen, prolactin, or growth hormone variant hGH-V.

A further aspect of the invention is an isolated nucleic acid encoding the anti-angiogenic peptide of the invention. The nucleic acid may be DNA or RNA, and may comprise cDNA sequences.

A further aspect of the invention is a host cell comprising the nucleic acid encoding the anti-angiogenic peptide of the invention. A further aspect of the invention is a method of producing the peptide of the invention, comprising expressing the nucleic acid in the host cell, and recovering the peptide.

A further aspect of the invention is a method of treating an angiogenic disease in a subject, the method comprising administering to a subject in need of such treatment an angiogenesis inhibitory effective amount of the antiangiogenic peptide of the invention.

A further aspect of the invention is a method of inhibiting tumor formation or growth in a patient, the method comprising administering to the patient an angiogenesis inhibitory effective amount of the antiangiogenic peptide of

the invention.

A further aspect of the invention is a method for diagnosing a probable abnormality of placental vascularization during pregnancy comprising assaying a level of at least one of endogenous N-terminal fragments of growth hormone, prolactin, growth hormone variant hGH-V, and placental lactogen in a tissue sample from a patient; and

5 comparing the level of the at least one of endogenous N-terminal fragments to an average level of the at least one of endogenous N-terminal fragments in a normal 10 patient population;

wherein a level of the at least one of endogenous N-terminal fragments higher than the average level is a probable abnormality of placental vascularization during pregnancy.

15 A further aspect of the invention is a method of modulating vascularization of a patient's placenta, the method comprising administering to the patient an angiogenesis inhibitory effective amount of the anti-angiogenic peptide of the invention.

20

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing *in vitro* antiangiogenic effect of peptides of the invention detected as an inhibition of bovine brain capillary endothelial cell proliferation.

25 Figure 2 is a graph of relative antiangiogenic activity in the chick chorioallantoic membrane of 16K N-terminal fragment peptides of human growth hormone, growth hormone variant hGH-V, human placental lactogen and human prolactin compared to their respective intact 22 or 23K polypeptides and to a bovine serum albumin control.

30 Figure 3 comprises Figures 3A-H, which are photographs of a subset of data from Figure 2 comparing *in vivo* neovascularization of the chick chorioallantoic membrane following the treatment with the peptides of the invention.

35 Figure 4 comprises Figures 4A-C. Figure 4A is a Western blot analysis of tyrosine phosphorylation of mitogen-activated protein kinase. Figure 4B is a graph depicting the percent level of inhibition of the bFGF stimulation of MAPK

enzymatic activity by 16K N-terminal fragments. Figure 3C is a graph depicting percent of stimulation over control by intact hormones without the presence of 16K fragment.

Figure 5 comprises Figures 5A-5B. Figure 5A depicts Western blot analysis of plasminogen activator inhibitor-1 protein levels and their quantitation following treatment with 16K N-terminal fragments or intact hormones. Figure 5B is a Western blot analysis of plasminogen activator inhibitor (PAI-1) in untreated cells and cells treated with 16K N-terminal hGH, hGH, or a combination thereof.

Figure 6 depicts a silver stained SDS-polyacrylamide gels showing cleavage of human prolactin by cathepsin D *in vitro*.

Figure 7 is Western blot of human amniotic fluid prolactin showing cleavage of human prolactin in amniotic fluid into fragments of 18 kD and 13 kD.

Figure 8 comprises Figures 8A and 8B and depicts HPLC fractions of human prolactin cleaved with cathepsin D (8A) and assayed for their ability to inhibit bFGF-induced bovine brain capillary endothelial (BBCE) cell  $^3\text{H}$ -thymidine incorporation (8B).

Figure 9 is a graph depicting increases in the number of 16K N-terminal hPRL binding sites in the human placenta throughout pregnancy expressed as specific binding.

25                   DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention provides novel antiangiogenic peptides which are potent inhibitors of angiogenesis. These peptides in general correspond to about 10 to about 150 consecutive amino acids, more preferably about 10 to about 100, most preferably about 10 to about 50 consecutive amino acids selected from the amino acid sequences of N-terminal fragments of about 13-16 kD ("16K") derived from full length human growth hormone, the growth hormone variant hGH-V, human placental lactogen or human prolactin. As defined, herein peptides "substantially identical" in amino acid sequence are also included in the scope in the invention. The about 13-16K N-terminal fragments of the full length hormones are also

referred to herein as "16K N-terminal human growth hormone" (16K hGH), "16K N-terminal human placental lactogen" (16K - hPL), "16K N-terminal growth hormone variant hGH-V" (16K hGH-V), and "16K N-terminal human prolactin" (16K hPRL). The 5 peptides of the invention have the properties of (i) inhibiting capillary endothelial cell proliferation and organization; (ii) inhibiting angiogenesis in chick chorioallantoic membrane; and (iii) binding to at least one specific receptor which does not bind an intact full length 10 growth hormone, placental lactogen or growth hormone variant hGH-V.

The finding disclosed herein that 16K N-terminal fragments cleaved from this group of hormones bind to at least one new, previously undisclosed receptor or receptors other 15 than their own specific receptors known in the art and have specific and distinguishable antiangiogenic activity is surprising and unexpected. The full length intact hormones have no antiangiogenic activity but are angiogenic in some settings. Furthermore, the antiangiogenic activity of the 16K 20 N-fragments is not mediated via the intact hormones' own specific receptors.

The peptides of the invention have similar biological potencies acting in the nM concentrations. Typically, one-half of the inhibitory action  $IA_{50}$  is achieved 25 at a very low concentration of about 0.8-1 nM. The peptides bind to the new receptor(s) with high affinity and in a saturable manner. Intact full length growth hormone, placental lactogen, growth hormone variant hGH-V, and bFGF do not compete for the same receptor site(s) and have different 30 activities as well as levels of activities.

In *in vitro* assays, the peptides of the invention inhibit the growth of bovine brain capillary endothelial (BBCE) cell proliferation confirming their antiangiogenic activity. Additionally, the peptides inhibit the mitogenic 35 activity of bFGF and VEGF. While 16K N-terminal human growth hormone has this activity, rat 16K N-terminal growth hormone is not antiangiogenic.

A. Angiogenesis, Angiogenic Conditions and Angiogenic Diseases

The peptides of the invention and their respective pharmaceutical compositions and preparations which are capable of inhibiting angiogenesis are useful for preventing or treating any disease or condition which is associated with or results in or from angiogenesis. Such diseases include formation of malignant tumors, angiofibroma, arteriovenous malformations, arthritis, such as rheumatoid arthritis, atherosclerotic plaques, corneal graft neovascularization, delayed wound healing, proliferative retinopathy such as diabetic retinopathy, macular degeneration, granulations such as those occurring in hemophilic joints, inappropriate vascularization in wound healing such as hypertrophic scars or keloid scars, neovascular glaucoma, ocular tumor, uveitis, non-union fractures, Osler-Weber syndrome, psoriasis, pyogenic glaucoma, retrobulbar fibroplasia, scleroderma, solid tumors, Kaposi's sarcoma, trachoma, vascular adhesions, chronic varicose ulcers, leukemia, and reproductive disorders such as follicular and luteal cysts and choriocarcinoma, among others.

Given their anti-angiogenic activity, the peptides of the invention are also suitable for use in a method of inhibiting mammalian cell proliferation and organization that depends on vascularization, including the selective inhibition of vascularization of tumors, tumor size reduction and elimination. Examples of tumors undergoing angiogenesis include but are not limited to angiofibroma, arteriovenous malformations, ocular tumors, all solid tumors, Kaposi's sarcoma, trachoma and choriocarcinoma.

The peptides of the invention may be used to assess and/or modulate the development of the vasculature of the placenta. Regulation of placental vascularization has important clinical implications, since two disorders of pregnancy, preeclampsia and intrauterine growth retardation, are associated with impairment of vascular development. No clinical tests exist to predict the occurrence of these disorders until pregnancy is seriously compromised.

The peptides of the invention can also be used as

contraceptive agents.

B. Diagnostic Assays for Placental Dysfunction and Abnormal Vascularization

Measurement of the endogenous levels of at least one of naturally occurring N-terminal fragments of hGH, hGH-V, hPRL, or hPL, such as 16K hGH, 16K hGH-V, 16K hPL or 16K hPRL, in the blood or tissue sample of a pregnant women by, for example, radioimmunoassay provides a diagnostic assay for the probable impairment of vascular development associated with preeclampsia, intrauterine growth retardation, and placental dysfunction. The activity level of enzymes which generate such fragments, such as but not limited to endogenous cathepsin, thrombin, plasmin, or subtilisin, can also be measured as an indication of probable vascular abnormalities in the placenta. Measurements are typically done in the first trimester, but may be performed in the second and/or third trimesters.

Any technique known in the art for detecting levels of peptides or polypeptides of known function or sequence may be used for the diagnostic assay. Typically, the diagnostic assay would comprise assaying a level of at least one of endogenous N-terminal fragments of prolactin, growth hormone, growth hormone variant hGH-V, and placental lactogen in a tissue sample from a patient; and comparing the level of the at least one of endogenous N-terminal fragments to an average level of the at least one of endogenous N-terminal fragments in a normal patient population; wherein a level of the at least one of endogenous N-terminal fragments higher than the average level is a probable abnormality of placental vascularization during pregnancy.

The phrase "specifically binds to an antibody" or "specifically immunoreactive with", when referring to a protein or peptide, refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a

significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

C. Production of the Peptides of the Invention

The peptides of the current invention can, for example, be synthesized, prepared from purified full-length hormones, or produced using recombinant methods and techniques known in the art. Although specific techniques for their preparation are described herein, it is to be understood that all appropriate techniques suitable for production of these peptides are intended to be within the scope of this invention.

Generally, these techniques include DNA and protein sequencing, cloning, expression and other recombinant engineering techniques permitting the construction of prokaryotic and eukaryotic vectors encoding and expressing each of the peptides of the invention.

In one mode, the peptides of this invention are conveniently obtained by isolation of intact growth hormone from the human pituitary gland or plasma and isolation of lactogen and growth hormone variant hGH-V. The isolated intact hormones may be glycosylated and cleaved to varying degrees.

In another mode, the peptides may be prepared by peptide synthesis according to method described in *Biotechnology and Applied Biochem.*, 12:436 (1990) or by methods described in *Current Protocols in Molecular Biology*, Eds. Ausubel, F.M., et al, John Wiley & Sons, N.Y. (1987).

The peptides of the invention may be produced by expression of a nucleic acid encoding a peptide of interest, or by cleavage from a longer length polypeptide encoded by the nucleic acid. Expression of the encoded polypeptides may be 5 done in bacterial, yeast, plant, insect, or mammalian hosts by techniques well known in the art. As for example, 16K hPRL has been produced in eukaryotic HCT 116 cells. The cDNA coding for the 16K hPRL (stop 140) has been cloned into the mammalian expression vector pRC-CMV. The construct was 10 transferred into the HCT116 human colon cancer cells and stably transfected cells expressing 16K hPRL (stop 140) were obtained. Either conditioned media or purified 16K hPRL (stop 140) from these cells were able to inhibit bFGF-induced BBCE proliferation.

15 In an embodiment, a peptide of interest of the invention is obtained by cloning the DNA sequence encoding an intact full length human hormone into a vector; modifying the DNA codon corresponding to the last amino acid of a desired 16K N-terminal hormone fragment to a stop codon by mutagenesis 20 techniques known in the art; and transforming a host cell with the modified nucleic acid to allow expression of the encoded peptide. In a further embodiment, the cloned hormone DNA is engineered to create a proteolytic cleavage site within the hormone polypeptide. The polypeptide is then cleaved after 25 production in the host to generate the peptide of interest. Examples of mutagenesis techniques include, for example, methods described in *Promega Protocols and Applications Guide*, Promega Corp, Madison, WI, p.98 (1891) or according to *Current Protocols in Molecular Biology*, *supra*.

30 If the peptide is to be synthesized in a prokaryotic vector, the DNA sequence encoding human hormone preferably does not contain a signal peptide sequence. In addition, a DNA codon for methionine (Met) is typically inserted upstream of 5' to the first DNA codon of the coding sequence.

35 Methods for cloning DNA into a vector and for inserting, deleting and modifying polynucleotides and for site directed mutagenesis are described, for example, in *Promega Protocols and Applications Guide*, *supra*. Cells or bacteria

may be transfected with a vector, preferably with an expression vector, having the desired DNA sequence attached thereto, by known techniques including heat shock, electroporation, calcium phosphate precipitation and lipofection, among others. The terminal peptides or other analogues or fragments may then be extracted and purified by, for example, high pressure liquid chromatography (HPLC), ion exchange chromatography or gel permeation chromatography. However, other methods and techniques known in the art of conducting the different steps or combinations of these steps necessary to derive the peptide of this invention or equivalent steps are contemplated to be within the scope of this invention.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, or may comprise a complete cDNA or gene sequence.

Optimal alignment of sequences for aligning a comparison window may, for example, be conducted by the local homology algorithm of Smith and Waterman Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson and Lipman Proc. Natl. Acad. Sci. U.S.A. 85:2444 (1988), or by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI).

As applied to polypeptides, the terms "substantial identity" or "substantial sequence identity" mean that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90

percent sequence identity, more preferably at least 95 percent sequence identity or more. "Percentage amino acid identity" or "percentage amino acid sequence identity" refers to a comparison of the amino acids of two polypeptides which, when optimally aligned, have approximately the designated percentage of the same amino acids. For example, "95% amino acid identity" refers to a comparison of the amino acids of two polypeptides which when optimally aligned have 95% amino acid identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to effect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.

The phrase "substantially purified" or "isolated" when referring to a peptide or protein, means a chemical composition which is essentially free of other cellular components. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein which is the predominant species present in a preparation is substantially purified. Generally, a substantially purified or isolated protein will comprise more than 80% of all macromolecular species present in the preparation. Preferably, the protein is purified to represent greater than 90% of all macromolecular species present. More preferably the protein is purified to greater than 95%, and most preferably the protein is purified to essential homogeneity, wherein other macromolecular species are not detected by conventional techniques.

#### D. Nucleic Acids of the Invention

Also provided herein are isolated nucleic acids that comprise DNA or RNA sequences encoding the peptides of the invention. The nucleic acids of the invention may further comprise vectors for expression of the peptides of the

invention. In some embodiments the DNA may comprise cDNA sequences encoding full-length hormones or sequences encoding N-terminal regions of the hormones. It is understood by one of ordinary skill in the art that because of degeneracy in the genetic code, substitutions in the nucleotide sequence may be made which do not result in changes in the encoded amino acid sequence. Thus, "substantially identical" sequences as defined herein are included in the scope of the invention. It is further understood by one of ordinary skill in the art that both complementary strands of any DNA molecule described herein are included within the scope of the invention.

The terms "substantial identity" or "substantial sequence identity" as applied to nucleic acid sequences and as used herein denote a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, and more preferably at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence.

#### E. Agonists

In some embodiments of the invention, agonists of the receptor(s) for the anti-angiogenic peptides are provided. Such agonists include but are not limited to mutants of the peptides of the invention and peptide, non-peptide, and peptidomimetic analogs of the peptides of the invention.

#### F. Treatment Protocols

The method for treatment of angiogenic diseases comprises administering to a patient an angiogenesis inhibitory amount of one or more peptides of the invention.

As used herein, the term "treatment" is intended to refer to the prevention, amelioration, or reduction in severity of a symptom of angiogenesis. Similarly, an angiogenic-inhibitory effective dose of a peptide of the invention is a dose sufficient to prevent, ameliorate, or reduce the severity of a symptom of angiogenesis.

The peptides of the invention may be administered singly or in combination with each other or other angiogenesis inhibitory agents.

Typically, the peptides of the invention are administered in an amount of about 8 micrograms to 3,000  $\mu\text{g}/\text{kg}$  per day, and more preferably about 20 to 1,500  $\mu\text{g}/\text{kg}$  per day preferably once or twice daily. However, other amounts, including substantially lower or higher amounts, may also be administered. The peptides of the invention are administered to a human subject in need of antiangiogenic treatment intramuscularly, subcutaneously, intravenously, intratumorally, by any other acceptable route of administration. In the case of ocular angiogenic diseases, the peptide may also be administered topically to the eye.

Both preventative or therapeutic uses, such as the prevention and/or treatment of diabetic patients to avoid a decrease of their vision produced by vascularization of the retina, contraceptive applications, and the long-term treatment of cancer patients such as for avoiding the reformation of malignant tumors after surgery or chemotherapy are intended.

As it is well-known that some patients, e.g. diabetic patients suffer from vision loss over a period of time, the present peptide may be suitably utilized for the inhibition or retardation of this process. When utilized for this application, the composition of the invention to be administered may comprise an amount of the peptide about 12 to 3,500  $\mu\text{g}/\text{kg}$  per day, and preferably about 25 to 2,700  $\mu\text{g}/\text{kg}$  per day. However, different amounts of the peptide may also be administered as seen suitable by a practitioner for specific cases. Smaller amounts may be administered by injection into the anterior chamber of the eye.

For this or any other this application the peptide of this invention may be administered in an amount of about 10 to 3,750 µg/kg, and more preferably about 15 to 1,600 µg/kg. Any means of administration is suitable.

5           G. Gene Therapy

Gene therapy utilizing recombinant DNA technology to deliver nucleic acids encoding peptides of the invention into patient cells or vectors which will supply the patient with gene product *in vivo* is also contemplated within the scope of 10 the present invention.

Gene therapy techniques have the potential for limiting the exposure of a subject to a gene product, such as polypeptide, by targeting the expression of the therapeutic gene to a tissue of interest, such as skeletal muscle, 15 myocardium, vascular endothelium or smooth muscle, or solid or circulating tumor cells. For example, WIPO Patent Application Publication No. WO 93/15609 discloses the delivery of interferon genes to vascular tissue by administration of such genes to areas of vessel wall injury using a catheter system. 20 In another example, an adenoviral vector encoding a protein capable of enzymatically converting a prodrug, a "suicide gene", and a gene encoding a cytokine are administered directly into a solid tumor.

Other methods of targeting therapeutic genes to 25 tissues of interest include the three general categories of transductional targeting, positional targeting, and transcriptional targeting (for a review, see, e.g., Miller et al. FASEB J. 9:190-199 (1995)). Transductional targeting refers to the selective entry into specific cells, achieved 30 primarily by selection of a receptor ligand. Positional targeting within the genome refers to integration into desirable loci, such as active regions of chromatin, or through homologous recombination with an endogenous nucleotide sequence such as a target gene. Transcriptional targeting 35 refers to selective expression attained by the incorporation of transcriptional promoters with highly specific regulation of gene expression tailored to the cells of interest.

Examples of tissue-specific promoters include a liver-specific promoter (Zou et al., Endocrinology 138:1771-1774 (1997)); a small intestine-specific promoter (Olivera et al., J. Biol. Chem. 271:31831-31838 (1996)); the promoter for creatine kinase, which has been used to direct the expression of dystrophin cDNA expression in muscle and cardiac tissue (Cox et al., Nature 364:725-729 (1993)); and immunoglobulin heavy or light chain promoters for the expression of suicide genes in B cells (Maxwell et al., Cancer Res. 51:4299-4304 (1991)). An endothelial cell-specific regulatory region has also been characterized (Jahroudi et al., Mol. Cell. Biol. 14:999-1008 (1994)). Amphotrophic retroviral vectors have been constructed carrying a herpes simplex virus thymidine kinase gene under the control of either the albumin or alpha-fetoprotein promoters (Huber et al., Proc. Natl. Acad. Sci. U.S.A. 88:8039-8043 (1991)) to target cells of liver lineage and hepatoma cells, respectively. Such tissue specific promoters can be used in retroviral vectors (Hartzoglu et al., J. Biol. Chem. 265:17285-17293 (1990)) and adenovirus vectors (Friedman et al., Mol. Cell. Biol. 6:3791-3797 (1986)) and still retain their tissue specificity.

Other elements aiding specificity of expression in a tissue of interest can include secretion leader sequences, enhancers, nuclear localization signals, endosmolytic peptides, etc. Preferably, these elements are derived from the tissue of interest to aid specificity.

Viral vector systems useful in the practice of the instant invention include but are not limited to adenovirus, herpesvirus, adeno-associated virus, minute virus of mice (MVM), HIV, sindbis virus, and retroviruses such as Rous sarcoma virus, and MoMLV. Typically, the nucleic acid encoding the therapeutic polypeptide or peptide of interest is inserted into such vectors to allow packaging of the nucleic acid, typically with accompanying viral DNA, infection of a sensitive host cell, and expression of the polypeptide or peptide of interest.

In still other embodiments of the invention, nucleic acid encoding a therapeutic polypeptide or peptide of interest

is conjugated to a cell receptor ligand for facilitated uptake (e.g., invagination of coated pits and internalization of the endosome) through a DNA linking moiety (Wu et al., J. Biol. Chem. 263:14621-14624 (1988); WO 92/06180). For example, the 5 DNA constructs of the invention can be linked through a polylysine moiety to asialo-oromucoid, which is a ligand for the asialoglycoprotein receptor of hepatocytes.

Similarly, viral envelopes used for packaging the recombinant constructs of the invention can be modified by the 10 addition of receptor ligands or antibodies specific for a receptor to permit receptor-mediated endocytosis into specific cells (e.g., WO 93/20221, WO 93/14188; WO 94/06923). In some embodiments of the invention, the DNA constructs of the invention are linked to viral proteins, such as adenovirus 15 particles, to facilitate endocytosis (Curiel et al., Proc. Natl. Acad. Sci. U.S.A. 88:8850-8854 (1991)). In other embodiments, molecular conjugates of the instant invention can include microtubule inhibitors (WO 94/06922); synthetic peptides mimicking influenza virus hemagglutinin (Plank et 20 al., J. Biol. Chem. 269:12918-12924 (1994)); and nuclear localization signals such as SV40 T antigen (WO 93/19768).

The nucleic acid can be introduced into the tissue of interest *in vivo* or *ex vivo* by a variety of methods. In some embodiments of the invention, the nucleic acid is 25 introduced to cells by such methods as microinjection, calcium phosphate precipitation, liposome fusion, or biolistics. In further embodiments, the nucleic acid is taken up directly by the tissue of interest. In other embodiments, nucleic acid is packaged into a viral vector system to facilitate introduction 30 into cells.

In some embodiments of the invention, the compositions of the invention are administered *ex vivo* to cells or tissues explanted from a patient, then returned to the patient. Examples of *ex vivo* administration of gene 35 therapy constructs include Arteaga et al., Cancer Research 56(5):1098-1103 (1996); Nolta et al., Proc Natl. Acad. Sci. USA 93(6):2414-9 (1996); Koc et al., Seminars in Oncology 23(1):46-65 (1996); Raper et al., Annals of Surgery 223(2):116-

26 (1996); Dalesandro et al., J. Thorac. Cardi. Surg.  
11(2):416-22 (1996); and Makarov et al., Proc. Natl. Acad.  
Sci. USA 93(1):402-6 (1996).

#### H. Formulations and Pharmaceutical Compositions

5 The compositions of the invention will be formulated for administration by manners known in the art acceptable for administration to a mammalian subject, preferably a human. In some embodiments of the invention, the compositions of the invention can be administered directly into a tissue by  
10 injection or into a blood vessel supplying the tissue of interest. In further embodiments of the invention the compositions of the invention are administered "locoregionally", i.e., intravesically, intralesionally, and/or topically. In other embodiments of the invention, the compositions of the invention are administered systemically by  
15 injection, inhalation, suppository, transdermal delivery, etc. In further embodiments of the invention, the compositions are administered through catheters or other devices to allow access to a remote tissue of interest, such as an internal  
20 organ. The compositions of the invention can also be administered in depot type devices, implants, or encapsulated formulations to allow slow or sustained release of the compositions.

In order to administer therapeutic agents based on,  
25 or derived from, the present invention, it will be appreciated that suitable carriers, excipients, and other agents may be incorporated into the formulations to provide improved transfer, delivery, tolerance, and the like.

A multitude of appropriate formulations can be found  
30 in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences, (15th Edition, Mack Publishing Company, Easton, Pennsylvania (1975)), particularly Chapter 87, by Blaug, Seymour, therein. These formulations include for example, powders, pastes, ointments, jelly, waxes,  
35 oils, lipids, anhydrous absorption bases, oil-in-water or water-in-oil emulsions, emulsions carbowax (polyethylene glycols of a variety of molecular weights), semi-solid gels,

and semi-solid mixtures containing carbowax.

Any of the foregoing formulations may be appropriate in treatments and therapies in accordance with the present invention, provided that the active agent in the formulation is not inactivated by the formulation and the formulation is physiologically compatible.

The quantities of active ingredient necessary for effective therapy will depend on many different factors, including means of administration, target site, physiological state of the patient, and other medicaments administered.

Thus, treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of the active ingredients. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage.

Various considerations are described, for example, in Goodman and Gilman's the Pharmacological Basis of Therapeutics, 7th Edition (1985), MacMillan Publishing Company, New York, and Remington's Pharmaceutical Sciences 18th Edition, (1990) Mack Publishing Co, Easton Penn. Methods for administration are discussed therein, including oral, intravenous, intraperitoneal, intramuscular, transdermal, nasal, iontophoretic administration, and the like.

The compositions of the invention may be administered in a variety of unit dosage forms depending on the method of administration. For example, unit dosage forms suitable for oral administration include solid dosage forms such as powder, tablets, pills, capsules, and dragees, and liquid dosage forms, such as elixirs, syrups, and suspensions. The active ingredients may also be administered parenterally in sterile liquid dosage forms. Gelatin capsules contain the active ingredient and as inactive ingredients powdered carriers, such as glucose, lactose, sucrose, mannitol, starch, cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate and the like. Examples of additional inactive ingredients that may be added to provide desirable color, taste, stability,

buffering capacity, dispersion or other known desirable features are red iron oxide, silica gel, sodium lauryl sulfate, titanium dioxide, edible white ink and the like. Similar diluents can be used to make compressed tablets. Both 5 tablets and capsules can be manufactured as sustained release products to provide for continuous release of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric-coated for selective 10 disintegration in the gastrointestinal tract. Liquid dosage forms for oral administration can contain coloring and flavoring to increase patient acceptance.

The concentration of the compositions of the invention in the pharmaceutical formulations can vary widely, 15 i.e., from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

20 The compositions of the invention may also be administered via liposomes. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the composition of the invention to be 25 delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to a desired target, such as antibody, or with other therapeutic or immunogenic compositions. Thus, liposomes either filled or decorated with a desired composition of the invention can delivered 30 systemically, or can be directed to a tissue of interest, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions.

Liposomes for use in the invention are formed from 35 standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety

of methods are available for preparing liposomes, as described in, e.g., Szoka et al. Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, incorporated herein by reference.

5 A liposome suspension containing a composition of the invention may be administered intravenously, locally, topically, etc. in a dose which varies according to, inter alia, the manner of administration, the composition of the invention being delivered, and the stage of the disease being treated.

10 For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral 15 administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or 20 more compositions of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the compositions of the invention are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of 25 compositions of the invention are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters 30 of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The 35 surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

The compositions of the invention can additionally be delivered in a depot-type system, an encapsulated form, or an implant by techniques well-known in the art. Similarly, the compositions can be delivered via a pump to a tissue of interest.

The compositions of the invention are typically administered to patients after the onset of symptoms, although treatment can also be prophylactic in some embodiments. Typically, treatment with direct administration of polypeptides is done daily, weekly, or monthly, for a period of time sufficient to reduce, prevent, or ameliorate symptoms. Treatment with the nucleic acids of the invention is typically done at intervals of several months. In some embodiments, administration of the compositions of the invention is done in utero.

The composition of the invention may also be provided in the kit as a slow-release composition such as a daily, weekly, monthly unit provided as a sponge, dermal patch, subcutaneous implant and the like in a wrapping or container as described above. In this case, the patient may release a unit of the composition from the container and applies it as indicated in the kit instructions. The composition may then be replaced at the end of the specified period by a fresh unit, and so on.

The present composition may also be administered by means of injection, as indicated above. Typically, the peptide may be administered by itself, or, for instance, in the case of a diabetic, in a composition also comprising insulin. The same is true for the slow-release forms of the composition. Similarly, the peptide of the invention may be administered in a composition that also comprises another drug. One such case is that of cancer patients, where different anti-cancer drugs such as chemotherapeutic or contrast agents and target-specific antibodies, among others, may be provided in a composition also comprising the peptide of the invention. The proportion of peptides to the other drug(s) and carrier may be adjusted accordingly.

The levels of the delivered peptide to a patient may

be monitored by immunoassay. To determine the level of the peptide of invention in blood following administration, e.g., intramuscular or subcutaneous administration, an antibody assay may be performed with antibodies specific to the peptide sequence by any of the protocols known in the art. Polyclonal or monoclonal antibodies or the 16K N-terminal fragment receptor may be utilized. The level of the peptide in blood may then be correlated with the progress of the inhibition of any of the diseases the patient is afflicted with.

10 The following examples are intended to illustrate, not limit the scope of this invention.

Example 1. Antiangiogenic Activity of 16K N-Terminal Fragments of Human Growth Hormone, Human Placental Lactogen, Human Prolactin, and Human Growth Hormone Variant hGH-V

15 16K N-terminal antiangiogenic agents were derived from full length human growth hormone, human growth hormone variant hGH-V, human placental lactogen and prolactin. These peptides were tested *in vivo* and *in vitro* for their binding and antiangiogenic activity in assays described in below.

20 Results of these testings are illustrated in Figures 1-5.

A. In Vitro and in Vivo Angiogenic Inhibitory Activity

25 Biological antiangiogenic activity of the 16K N-terminal fragments was tested in bovine brain capillary endothelial cell assay, as well as *in vivo* chick chorioallantoic membrane assay. The *in vitro* and *in vivo* antiangiogenic inhibitory characteristics of the peptides of the invention were determined and are described in Sections 1 and 2, below.

30 1. In vitro Antiangiogenic Activity

In *vitro*, the peptides were tested for their ability to inhibit the basal or stimulated growth of bovine brain capillary endothelial cells. Fibroblast growth factors were used to stimulate endothelial cell growth. Experimental procedures for these tests are described in below.

35 *In vitro* inhibition of bFGF stimulation of bovine

brain capillary endothelial (BBCE) cell proliferation is shown in Figure 1.

Figure 1 is a graph showing inhibition of bovine brain capillary endothelial cell proliferation expressed as the percent of inhibition of the stimulation by bFGF.

Experimental design for studies seen in Figure 1 was as follows. On day 1,  $10^4$  bovine brain capillary endothelial (BBCE) cells were plated in 24-well plates in 0.25 ml Dulbecco's modified Eagle's medium obtained from Gibco, containing 10% fetal calf serum, human recombinant bFGF (1 ng/ml, Promega), with purified peptide (100, 50, 10, 5, 2, 1, 0.5, 0.2, or 0.1 nM) prepared as discussed below. Wells containing cells, medium and serum without presence of angiogenesis stimulator basic fibroblast growth factor (bFGF) were included as basal-growth controls. On day 3, bFGF (1 ng/ml) and 100, 50, 10, 5, 2, 1, 0.5, 0.2, or 0.1 nM purified protein were added once again to the dishes. On day 4, the cells were incubated with 500,000 cpm of (<sup>3</sup>H) thymidine for 4 hours, washed in 5% trichloroacetic acid, solubilized in NaOH, and counted as previously described in *Endocrinology*, 133: 1292 (1993). Each point seen in Figure 1 represents the mean of triplicate wells. The experiments were repeated at least three times. Similar results were obtained in each experiment.

The data seen in Figure 1 are expressed as percentages of the inhibition of the stimulation obtained with bFGF alone which was considered to be 100% when the hormone concentration was less than 0.1 nM, 0% being the basal growth level.

As seen from Figure 1, when the concentration of the hormone peptide was zero or lower than 0.1 nM, there was 100% bFGF induced BBCE cell proliferation that was 100% angiogenic. However, following the treatment with 0.2 nM 16K hGH-V or other 16K fragments, the drop in bFGF stimulation was observed. When the concentration of the 16K peptides was increased to 1 nM, the drop in bFGF stimulation was substantial from 100 to about 60%. At 10 nM concentration of the 16K peptides, the bFGF-stimulation dropped to about 10-

20%. When the concentration of the 16K peptides was increased to about 50 nM, the bFGF stimulation of BBCE cell proliferation was completely suppressed.

Figure 1, therefore, clearly shows that purified 16K hormone fragments of the invention are able to substantially inhibit the cell proliferation even in concentrations as low as 1 nM. At concentrations from 10-50 nM, these peptides completely inhibit endothelial cell proliferation *in vitro*. Symbols used for individual 16K peptides are: (■) 16K hPRL; (◆) 16K hPL; (●) 16K hGH; (▲) 16K hGH-V.

As seen in Figure 1, *in vitro* 16K hPL, 16K hGH, and 16K hGH-V fragments and 16K hPRL used as a comparative control administered in concentrations from 0.1 to 100 nM almost completely inhibit induced basic fibroblast growth factor (bFGF) proliferation of bovine brain capillary endothelial (BBCE) cells. Inhibition of cell proliferation was dose dependent and the concentration required for half maximal inhibition ( $IC_{50}$ ) ranged from 1 to 5 nM for the various 16K N-terminal fragment peptides.

## 20           2. In vivo Antiangiogenic Activity

25           In *vivo*, the peptides of the invention were tested for their ability to inhibit basal or stimulated capillary formation in developing chick embryos, using the chick chorioallantoic membrane assay (CAM) described below. The ability to inhibit the development of capillaries in the chick chorioallantoic membrane is a measure of *in vivo* antiangiogenic activity. The peptides were applied to the chorioallantoic membrane by local placement of methylcellulose disks containing the peptide.

30           In *vivo* studies using chick chorioallantoic membrane (CAM) assay are shown in Figures 2 and 3.

35           Figure 2 shows the antiangiogenic effect of the peptides of the invention calculated as the percentage of eggs showing anti-angiogenic response in the CAM assay. The procedure is described below.

Figure 2 compares antiangiogenic activity in the chick chorioallantoic membranes assay of the intact full length hPRL, hGH, hGH-V and hPL to the antiangiogenic activity

of their respective 16K N-terminal fragment peptides.

The antiangiogenic activity is described as antiangiogenic index expressed in percent. Bovine serum albumin (BSA) was used as a protein control.

As seen from Figure 2, neither BSA nor any of the full length intact hormones had any antiangiogenic effect. Of the 16K N-terminal fragment peptides, the highest antiangiogenic index was observed for 16K hPRL and for 16 K hGH, both having antiangiogenic index close to 100%. The antiangiogenic index of 16K hGH-V was between 80-85% and antiangiogenic index of 16 K hPL was around 90%.

These findings show that antiangiogenic activity of 16K N-terminal fragments studied is specific to the N-terminal region of the full hormones and the locus of this activity is located within about 16K of the N-terminus. These findings are unexpected and surprising because the full length hormones give no indication of any such activity.

Figure 3 shows photographs of results of the chick chorioallantoic membrane (CAM) assay following the treatment with 16K hGH, 16K hGH-V, 16K hPL and 16K hPRL compared to their respective full length hormones and to a bovine serum albumin control. Figure 3 describes the inhibition of angiogenesis in early stage CAM assay.

For this study, on day 3 of development, fertilized chick embryos were removed from their shells and placed in plastic Petri dishes. On day 6, 5 mm disks of methylcellulose (0.5%, Sigma) containing 20 µg of 16K N-terminal fragments or intact molecules and 2 µg bovine serum albumin (BSA) were laid on the advancing edge of the chick CAM, as described below. After a 48-hour exposure, white India ink was injected into the chorioallantoic sac for photographic purposes. Under these conditions, the disks are visible by light reflection and the black arrow delineates the border of the disk or the border of the avascular area, if present.

Figure 3 shows photographs of neovascularization of chicken embryo subjected to treatment with BSA, (upper panel 1) serving as a protein control, to treatment with 16K peptides of the invention (upper panels 2-5) and to the

treatment with intact full length hormones (lower panels 6-9). The sites of the disks, identified as black arrow, show clearly that at sites of treatment with 16K N-terminal fragments, the neovascularization is almost completely or substantially suppressed. In lower panels, which show results obtained following the treatment with intact full length hormones high neovascularization is clearly visible. Such high neovascularization is completely unobservable in embryos treated with the 16K peptides of the invention. In *in vivo* CAM assays 16K hPRL, 16K hPL, 16K hGH and 16K hGH-V inhibited capillary formation as seen in Figures 2 and 3, confirming their antiangiogenic activity. Large areas devoid of capillaries were observed surrounding the methylcellulose disks containing the 16K fragments. No such activity was observed when cellulose disks contained BSA or the intact full length hormone molecules.

These findings show that the peptides of the invention inhibit both cell proliferation *in vitro* and also inhibit neovascularization *in vivo*.

Quantitation of the *in vivo* antiangiogenic effect of the peptides of the invention expressed as antiangiogenesis index is seen in Figure 2.

In view of the above described *in vitro* and *in vivo* results which indicate that the 16K N-terminal fragments of hPRL, hGH, hPL, and hGH-V hormones possess different biological properties than the intact full length hormones from which they are derived, the studies were performed to determine whether these peptides act on separate receptors.

For this purpose, a series of studies was performed to investigate the effects of the 16K N-terminal fragment peptides as well as 16K fragment of human prolactin on activation of mitogen-activated protein kinase (MAPK) in bovine brain capillary endothelial (BBCE) cells.

Both basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are known mitogens which activate the so called mitogen-activated protein kinase (MAPK) activity. The activity of MAPK, therefore, is a good indicator of mitogenic activity or its inhibition. Kinase

activity can be indirectly assessed by determining the level of tyrosine phosphorylation with a phosphotyrosine antibody or directly by measuring phosphorylation of a substrate.

The activation of MAPK was assessed indirectly by Western analysis using a phosphotyrosine antibody and directly by both *in vitro* and *in gel* enzymatic assays. Results are seen in Figure 4.

Figure 4 represents a Western blot analysis of tyrosine phosphorylation of MAPK (Figure 4A), and the percentage level of inhibition of the bFGF stimulation of MAPK enzymatic activity by 16K fragments (Figure 4B) compared to % of stimulation over control without 16K fragment (Figure 4C). Stimulation of the tyrosine phosphorylation of MAPK by bFGF was blocked by the addition of 16K hGH, 16K hGH-V and 16K hPL as well as 16K hPRL (Figure 4C).

As seen in Figure 4, 16K fragments inhibit bFGF-dependent MAPK tyrosine phosphorylation and activity, while full-length hormones stimulate these processes in the absence of bFGF. For this study, cell lysates and phosphotyrosine Western blots were prepared and *in-gel* MAPK activity was measured as previously described in *EMBO J.*, 2:493 (1983). BBCE cells ( $10^5$ ) were plated in 60-mm plates in 1 ml Dulbecco's modified Eagle's medium (DMEM) Gibco, containing 10% calf serum. Twenty-four hours after plating, the cells were transferred to DMEM containing 0.5% calf serum and incubated for 48 hours to induce quiescence. The cells were treated for 5 min with 10 nM of the purified 16K hGH, 16K hGH-V, 16K hPL or 16K hPRL and with 250 pM bFGF, or for 10 minutes with 10 nM intact full length corresponding hormone without bFGF. Control was left untreated. To terminate the incubation, the medium was removed by suction and the cells were washed twice with phosphate buffer solution (PBS). Then 250  $\mu$ l lysis buffer containing 20 mM Tris-HCl pH 8; 137 mM NaCl; 10% glycerol; 1% Triton X100; 1 mM orthovanadate and protease inhibitors leupeptin (2 $\mu$ M), aprotinin (0.14  $\mu$ /ml) Pefablock (1 mM) was added to the plates and the samples were shaken at 4°C for 20 minutes.

Figure 4A, P-Tyr panel - left side panel shows the

inhibition of the bFGF-induced phosphorylation of the p42 and p44 MAPK which are 42 and 44K mitogen-activated kinases, respectively, also called erk-1 and erk-2. Right side panel shows the stimulation of the MAPK by the full length hormones in the absence of bFGF. Cell lysate proteins were separated by SDS-PAGE, transferred to PVDF membranes, and probed with an anti-phosphotyrosine antibody (4G10, UBI, 1/2000 dilution). In Figure 4A MAPK panel was used to verify the identity of the tyrosine phosphorylated bands and to control protein loading, the Western Blot shown in the P-Tyr Panel was stripped and reprobed with an anti-MAPK antibody (erk: 1 ct, UBI, 1/10,000 dilution).

Level of inhibition of the stimulation of MAPK enzymatic activity by 16K N-terminal fragments of the invention is seen in Figure 4B. Results are compared to their respective intact hormone controls (Figure 4C).

Figures 4B and 4C show in-gel MAPK activity. For this study, cell lysates were resolved by SDS-PAGE in gels containing 0.5 mg/ml myelin basic protein (MBP) as discussed below. After electrophoreses, the gel proteins were denatured, renatured, and subjected to an *in situ* kinase reaction with 100  $\mu$ Ci of ( $\gamma$ - $^{32}$ ) ATP. The gels were then dried and autoradiographed.  $^{32}$ P incorporated into MBP was quantified by a phosphorimager. Numbers at the bottom of the histograms represent the number of experiments performed.

Figures 4B and 4C show comparative results of MAPK activity, expressed as percent of bFGF stimulation. The bFGF stimulation of MAPK activity was 100%. When the samples were submitted to treatment with both bFGF and 16K N-terminal fragment peptides of the invention, the bFGF-induced MAPK activity was inhibited in all cases to less than 25%, with greatest suppression of MAPK activity observed after treatment with the 16K hGH suppressing the MAPK activity to about 10%.

Figure 4C shows the stimulation of MAPK activity with intact full length hormone in the absence of bFGF. As seen from comparison of Figures 5B and 5C when the full length hormones were used instead of 16K N-terminal fragments, the intact molecules seen in Figure 4C actually stimulated the

tyrosine phosphorylation of MAPK. The inhibition of bFGF-stimulated MAPK enzymatic activity by the 16K N-terminal fragments ranged between 75 and 85% (Figure 4B), while the intact molecules actually stimulated MAPK activity.

5 The inhibition of the bFGF-induced stimulation of tyrosine phosphorylation and activation of MAPK by the 16K N-terminal fragments in contrast to the stimulations by the intact hormones clearly shows that the intact full length hormone and their 16K N-terminal fragments act on different  
10 receptors and have opposite effects on MAPK activation.

The above discussed results were further confirmed by the study performed to determine the effect of the peptide of the invention on the expression of plasminogen activator inhibitor (PAI). Secretion of type-1 plasminogen activator inhibitor (PAI-1) by migrating endothelial cells limits the degradation by urokinase plasminogen activator (uPA) of the extracellular matrix and thus prevents angiogenesis.  
15 Determination of whether or not the 16K peptides stimulate the expression of PAI-1 was performed and is described in Figure  
20 5.

Figure 5 is a Western blot analysis of the effect of the peptides of the invention on PAI-1 protein levels and their quantitation (Figure 5A) and Western blot analysis of PAI-1 in untreated cells and cells treated with hGH, 16K hGH,  
25 or both (Figure 5B).

For this study, cell lysates were prepared as described in *EMBO J.*, 2: 493 (1985). BBCE ( $10^5$ ) cells were plated in 24-well plates in 1 ml Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Twenty-four hours after plating, the cells were treated for 16 hours with purified 16K fragments or full-length hormone (10 nM) in serum-free DMEM. Untreated well was used as a control. 20  $\mu$ l cell lysate was resolved by SDS-PAGE (10-4%) and the separated proteins transferred to a nitrocellulose membrane. The blots  
30 were blocked for 1 hour with 5% milk in Tris-buffered saline with 0.1% Tween 20 and probed for 2 hours with mouse anti-bovine PAI-1 monoclonal antibody obtained from Gibco, at  
35 1:2000 dilution. The antigen-antibody complex was detected

with horseradish-peroxidase-conjugated secondary antibody and an enhanced chemiluminescence system (ECL, Amersham).

Figure 5A shows the effect of 16K N-terminal peptides of the invention on the expression of PAI-1 compared to the effect of intact hormones. Figure 5A shows that 16K fragments stimulate PAI-1 expression, whereas the full length hormones have no effect. PAI-1 stimulation induced by 16K fragments, expressed as fold stimulation, clearly shows that all four 16K peptides stimulate PAI-1 expression about 4 times that of control levels or levels observed following treatment with the intact hormones.

In the competition experiment shown in Figure 5B, the effects of 16K hGH (10 nM), intact hGH (50nM), and a mixture of 16K hGH and hGH were compared with controls. As seen from Figure 5B, treatment with intact hGH resulted in no stimulation of PAI-1 relative to the control. Treatment with 16K hGH alone stimulated PAI-1 expression while additions of intact hGH had no effect on the activity of 16K hGH.

As seen in Figure 5A, treatment of BBCE cells for 16 hours with each of the 16K fragments stimulated the expression of PAI-1 by approximately 300%. Treatment with their corresponding intact molecules had no effect on the expression of PAI-1, nor did addition of a five fold excess of hGH affect the stimulatory action of 16K hGH as seen in Figure 5B.

These data further support the conclusion that the 16K fragments signal through a different receptor than intact molecules. These data also clearly show that the 16K fragments are not acting as antiangiogenic factors via the bFGF receptors since the activity of the PAI-1 is independent of the action of bFGF.

Example 2. Endogenous 16K N-terminal fragments in the placenta

The interaction of fetal and maternal cells at the placental-decidua interface in eutherian mammals has been the subject of intensive investigation during the last two decades. Variation among species is dramatic. The most complex interactions are observed in hemochorial placentation,

e.g. in human and mouse, where fetal trophoblast cells are in direct contact with the maternal circulation.

The establishment of adequate placental vascularization is critical to the survival of the developing conceptus, and requires extensive proliferation and remodeling of the decidual vascular tree. If vasculogenic mechanisms of placentation are similar to those reported for tumor invasion there are at least two forces driving the acquisition of the new blood supply. Maternal endothelial cells are recruited toward the placenta and concomitantly, placental cells migrate toward proliferating maternal vessels.

The factors that prepare the endometrial capillary bed to allow implantation and subsequent placental vascularization are virtually unknown (*Obstet. Gynecol. Surv.*, 15 50:668-97 (1995)), yet these are likely to be under exquisite regulation. Failure of adequate placental vascularization is believed to be the primary pathogenetic defect in two common human pregnancy complications, namely in preeclampsia (*Ann. Med.*, 25:243-9 (1993)) and in intrauterine fetal growth 20 restriction (IUGR) (*Am. J. Obstet. Gynecol.*, 170:838-841 (1994)). By contrast, excessive trophoblastic invasion is associated with placenta accreta and gestational trophoblastic neoplasia.

Attachment of the implanting human placenta to the 25 maternal decidua is achieved by contact between anchoring villi of the chorion frondosum and the decidua basalis. The characteristic hemochorial vascular anatomy of the human placenta provides a unique environment for oxygen, nutrient and waste product exchange. Irregular lobes or cotyledons 30 separated by septa demarcate the maternal surface of the developing placenta. Normally, each cotyledon is supplied by one of approximately 120 maternal uterine spiral arteries.

The classical radiocontrast studies described in 35 *Contrib. Embryol.*, 38:59-70 (1966) revealed that maternal arterial blood is propelled through patent arteries toward the chorionic plate and disperses laterally through a complex system of vascular lacunae within the intervillous space that contains arterial, venous and capillary-like zones. In two

clinical conditions associated with fetal and placental hypoxia and vascular insufficiency, i.e. preeclampsia and IUGR, the number of patent spiral arteries is reduced (*Am. J. Obstet. Gynecol.*, 155: 401-12 (1986)). Pathological changes in this vasculature extend from the intervillous space to the base of the spiral arteries and may be due to the failure of normal angiogenic development.

The precise regulation of angiogenic factors expressed at the deciduoplacental interface is unknown currently but bFGF, VEGF (*Biol. Reprod.*, 51:524-30 (1994)) and other angiogenic proteins have been identified in human placenta. Clinical conditions associated with excessive trophoblastic invasion and neovascularity (e.g., placenta accreta, increta, precreta and choriocarcinoma), therefore, may be due to inadequate restraint by antiangiogenic agents. Conversely, premature or excessive production of antiangiogenic proteins relative to angiogenic factors could lead to poor maternal vascularization of the placental bed and result in fetoplacental ischemia as observed in preeclampsia and IUGR (*Clin. Perinatol.*, 18:661-82 (1994)). Based on the current findings, antiangiogenic peptides of the invention seem also to play a critical role in the human placenta, serving to regulate placental vascularization during normal pregnancy.

Recent reports have documented the expression of three angiogenic factors during early human placental development. VEGF is synthesized by human endometrium during the preimplantation phase (*J. Clin. Endocrinol. Metab.*, 81: 3112-18 (1996)), in the maternal decidua (*Growth Factors*, 12:235-43 (1995)) and by fetal trophoblast cells (*Biol. Reprod.*, 51:524-30 (1994)). Platelet-derived endothelial cell growth factor (PDGF) is expressed in secretory endometrium and decidua (*Human Reprod.*, 10:989-93 (1995)). Basic FGF is synthesized in endometrium and a preliminary report that bFGF is expressed throughout human placental development published in *Endocrine Society Meeting*, Abstract R35-4 (1997), confirms the current findings.

Normal placental neovascularization is most probably

controlled by the co-regulation of angiogenic and antiangiogenic factors. Dysregulations of the balance between these factors leads to hypovascularized placental conditions and subsequent pregnancy pathology. Three proteins expressed at the human placental-decidua interface would typically be candidates for antiangiogenic factors. Without being limited to any one theory, these are genetically related lactogenic proteins. These genes all encode primary protein products of about 20-25K. As documented above, however, these about 20-25K proteins possess no antiangiogenic activity. Each of these proteins, nevertheless, possesses peptidase recognition sequences that predict the possibility of their cleavage to 16K N-terminal fragments which have been now found to have antiangiogenic activity.

The following experiments were undertaken to test the hypothesis that 16K hPRL, 16K hPL and 16K hGH-V fragments of the lactogenic hormone family are spontaneously produced and play a physiological role in the vascular development of the human placenta.

A. Cathepsin D Cleavage of hPRL

The first question in developing the diagnostic assay was to determine whether full length hPRL could be enzymatically cleaved to generate a biologically active 16K fragment. Cathepsin-like enzymes, which have been shown to 25 cleave rat PRL to the 16K isoform (*Endocrinology*, 133:935-38 (1993)), e.g., cathepsin D, are expressed in several cells and tissues at the maternal-fetal interface. As shown herein, this enzyme can cleave human decidual PRL and can generate bioactive antiangiogenic fragments of purified hPRL.

Purified, intact full length 23K pituitary hPRL (30 µg) was incubated at 37°C in glycine-HCl buffer (pH 3.5) with human cathepsin D (0.3 µg). Aliquots of the incubation mix were taken at 0, 10, 20, 30, 60, 120 minutes and 24 hours. Samples were reduced with 5% β-mercaptoethanol, 35 electrophoresed on 13.5% SDS-polyacrylamide gels and silver stained.

Under these conditions, pituitary hPRL was cleaved

by cathepsin D, generating several peptides, as seen in Figure 6. The major fragment had an apparent molecular mass of about 13K with a minor fragment of about 18K. Under the same conditions, rat prolactin (rPRL) was cleaved into two major peptides of 16K and 8K as described previously (*Endocrinology*, 133:935-38 (1993)).

Next, a study was performed to confirm that human amniotic fluid prolactin, derived from the adjacent decidua, could be cleaved by endogenous protease(s) under acidic conditions to yield hPRL fragments identical to those generated by cathepsin D digestion *in vitro*, i.e. 13K, 18K or 16K.

Midtrimester amniotic fluid samples from 6 different subjects were adjusted to different pH ranging from 7.4 to 15 3.5, and incubated at 37°C for 24 hours. Western blotting with anti-prolactin antibody revealed immunoreactive bands representing undigested 23K PRL at pH 7.4, but when incubated under acidic conditions, two fragments of 18K and 13K were observed, as seen in Figure 7. The similarity of this pattern 20 with that generated by cathepsin D digestion suggested that endogenous cathepsin D might be responsible for this observation. Data indicate that endogenous PRL in late first trimester human decidual cytosol also can be cleaved by exogenous cathepsin D. Western blotting of non-reducing gels 25 indicated that endogenous decidual thiol reductase(s) reduce the 18K and 13K fragments under these conditions.

The pH optimum of hPRL cleavage by cathepsin D was then examined in more detail. Human PRL was cleaved *in vitro* by cathepsin D at pH 7.4, 5.5 and 3.5, reduced with β-30 mercaptoethanol and tested for antiangiogenic activity on BBCE cells. bFGF-induced mitogenesis of BBCE cells was inhibited 50% by hPRL cleaved at pH 7.4 and 100% by hPRL cleaved at pH 5.5 and 3.5. Intact full length hPRL has no effect on cell growth. Thus, while less efficient than under acidic 35 conditions, cathepsin D at physiological pH can generate bioactive, antiangiogenic fragments of hPRL.

B. Mapping of Cathepsin D Cleavage Site

To identify the cathepsin D cleavage site, recombinant 23K hPRL was treated as before with cathepsin D and the fragments resulting from the digestion were separated from intact PRL by gel filtration using a BioRad Biogel P-30 packed column. The column was equilibrated in 0.1 M ammonium bicarbonate buffer containing 200 mM  $\beta$ -mercaptoethanol (Endocrinology, 122:2892-98 (1988)). The PRL fragments were examined by SDS-PAGE using silver staining and revealed 18K and 16K species. The hPRL fragments were purified on a C18 reverse phase column. The fragments were reduced, loaded onto the column at pH=3.0 using 1% (v/v) TFA, and diluted using a gradient of acetonitrile containing 0.1% TFA. The purified fragments were separated on 13.5% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes in 10 mM CAPS (3-cyclohexylamino-1-propanesulfonic acid) buffer hP=11.0 with 10% methanol. Transferred protein was stained with 0.1% Coomassie blue, the two bands of interest, namely 18K and 13K, excised from the PVDF membrane and the peptides were sequenced directly on a gas phase Beckman-Porton PI2090 protein sequencer by Edman degradation sequencing to analyze the NH<sub>2</sub>-termini of the cleaved fragments.

As in the case of rat PRL where cleavage occurs at two sites, two cathepsin D cleavage sites between amino acids 36/37 and 133/134 of the mature hPRL were found. These sites resulted in two fragments, hPRL fragment 1-133 and hPRL fragment 37-133, consistent with the 18K and 13K bands identified in the corresponding Western blots of cleaved hPRL.

#### C. Biological Activity of Cathepsin D Cleaved hPRL

Separating the 18K and 13K fragments generated by cathepsin D cleavage of hPRL by HPLC was not successful, and both were eluted in fraction 6 seen in Figure 8A. Each of the HPLC fractions was assayed for their ability to inhibit bFGF-induced BBCE cell (<sup>3</sup>H)thymidine incorporation. Only fraction 6, containing the 18K and 13K fragments, inhibited the mitogenic response to exogenous bFGF, as seen in Figure 8B. Thus the mixture of the unseparated 18K and 13K PRL was responsible for the observed antiangiogenic activity.

D. Identification of Cathepsin D at the Human Deciduoplacental Interface

To establish the present of human cathepsin D mRNA in human placental and decidual tissue oligonucleotide primers that span exon 8 and 9 based on the human cathepsin D gene sequence (*DNA Cell Biol.*, 10:423-31 (1991)) were prepared. Total mRNA from these tissues was reverse transcribed and amplified using methods according to *J. Clin. Endocrine Metab.* 78:642-649 (1994). The results indicate that human placenta, endometrium and human umbilical vein endothelial cells all express cathepsin D mRNA transcripts (data not shown). Human fetal liver was used as a positive control. These results were extended by Western blotting of tissue lysates and biological fluids from pregnant and nonpregnant women. In reducing SDS-PAGE, anti-human cathepsin D antiserum detected the two subunits, about 25K and about 22K protein, in amniotic fluid, pregnancy plasma, and placental and decidual lysates. Nonpregnant endometrial lysates also contained cathepsin D protein.

E. Identification of Cathepsin D by Immunohistochemistry

Human fetal membranes and underlying decidua at term were evaluated by immunohistochemistry (IHC) using an antibody to human cathepsin D. Cathepsin D positive cells were observed in the amniotic membrane, chorionic trophoblast cells and scattered cells within the decidua. In human pregnancy the decidua synthesizes and secretes intact 23K hPRL into the extracellular space and the amniotic cavity. It has been confirmed that at least one enzyme capable of cleaving 23K PRL into antiangiogenic 16K fragments, cathepsin D, is not only synthesized locally at the deciduoplacental interface but also is present in amniotic fluid, plasma and in fact is synthesized by human endothelial cells. Thus, the necessary molecular components required to generate 16K PRL are available at the site of placental vascularization. The activity level of cathepsin D and/or concentrations of 16K fragments of hPRL, hPL and hGH-V are thus advantageously

utilized for detection of placental function, whether normal or abnormal. Besides the cathepsin D site in hPRL several other cleavage sites exist within the sequences of hGH, hGH-V and hPL that generate a N-terminal fragment of around 16 kDa.

5 hGH can be cleaved by plasmin, thrombin and subtilisin (EMBO J. 2:493 (1983)). The consensus cleavage sequence for these enzymes is also present in hGH-V and we have shown herein that hGH-V is cleaved by thrombin. Furthermore, preeclampsia has been characterized as a disease of an excess in thrombin

10 activity. Finally, hPL can be cleaved by plasmin to yield a 16K N-terminal fragment (J. Biol. Chem. 254:2296 (1979)).

F. 16K hPRL binding sites are present in human placenta increases throughout pregnancy

A novel, high affinity ( $K_d = 1$  nM) receptor for rat 16K PRL (16K rPRL) was described in capillary endothelial cells by radioligand binding studies with  $^{125}\text{I}$ -16K rPRL (Endocrinology, 130: 1380 (1992)). Utilizing this approach we measured specific 16K hPRL binding sites in human placental membrane fractions obtained at different stages of gestation.

15 By Scatchard analysis the dissociation constants were not significantly different in first, second or third trimester placental membranes. However, the maximal binding ( $B_{max}$ ) for membranes obtained during the first trimester was only 3.19 fmol/ $\mu\text{g}$  protein, whereas the  $B_{max}$  for the second trimester was 630 fmol/ $\mu\text{g}$  protein and 775 fmol/ $\mu\text{g}$  protein for the third trimester. These differences were confirmed by measurement of specific binding in multiple samples (Figure 9). Membranes from first trimester placentas ( $n=4$ ) had maximal specific binding of  $357 \pm 142.7$  cpm/ $5\mu\text{g}$  protein, while membranes from

20 second ( $n=3$ ) had  $1778 \pm 128$  cpm/ $5\mu\text{g}$  protein and third trimester ( $n=3$ ) had  $4041 \pm 1882$  cpm/ $5\mu\text{g}$  protein. These results support the hypothesis that 16K hPRL may play a role in the inhibition of angiogenesis following completion of the deciduoplacental interface. The absence of receptors early in pregnancy is

25 consistent with the idea that the action of an antiangiogenic factor would be inappropriate in early development and lead to inhibition of the normal vascular connection. Overproduction

of this antiangiogenic factor could be involved in disorders of pregnancy in which formation of the vascular connection of the placenta is compromised, e.g., preeclampsia.

Specimens of human placental tissues of first, second, and third trimester pregnancies were obtained from consenting women either undergoing elective pregnancy terminations (first and second trimester) or at the end of pregnancy (third trimester). The protocols were approved by the UCSF committee of human research. Tissues were kept at - 80°C until further processing. Rinsed tissues were homogenized in 1:1 volume of 20 mM Tris, pH 7.4, 250 mM sucrose, 2mM EDTA, and 0.2 mM Pefablock and centrifuged at 10,000 g for 30 min. The pellet was resuspended in 5 ml of buffer and recentrifuged at 10,000 g for 30 min. Both supernatants were brought to 100 mM NaCl and 0.2 mM MgCl<sub>2</sub> to precipitate microsomal membranes. The pellet from a subsequent centrifugation at 30,000 g for 45 min was resuspended in 20 mM Hepes, 10% glycerol, 1mM EDTA, 1mM Pefablock, and 0.14IU of aprotinin.

5 μg of the above membrane preparation were incubated with 100 pM of <sup>125</sup>I-16K rPRL with or without 400 nM of 16K hPRL in a final volume of 300 μl of assay buffer (100 mM Tris, 500 mM acetic acid, 10mM MgCl<sub>2</sub>, 0.1% BSA, pH 7.4) for 5h at room temperature. Assays were terminated by dilution with 3 ml cold assay buffer, followed by centrifugation at 3000 rpm for 45 min. at 4 °C and aspiration of supernants. Specific binding was determined by subtracting counts bound in the presence of an excess (400 nM) of unlabeled 16K hPRL from counts bound in the absence of unlabeled hormone.

Example 3. General Procedure for the Production of Peptides

This example illustrates the general methods used for the production of peptides of the invention.

5 Intact full length human prolactin (hPRL), human growth hormone (hGH), human growth hormone variant (hGH-V), human placental lactogen (hPL) and 16K N-terminal fragment of human prolactin (16K hPRL), growth hormone (16K hGH), growth hormone variant (16K hGH-V) and placental lactogen (16K hPL)  
10 were produced as described below.

The coding sequences, antisense sequences, and amino acid sequences of the intact hormones and 16K N-terminal peptides are listed in sequences identified as SEQ ID NO:1-30 as follows.

15 hPRL (Met<sup>-1</sup>Cys<sup>199</sup>) (SEQ ID NO:1)

ATGTTGCCCA TCTGTCCCGG CGGGGCTGCC CGATGCCAGG TGACCCCTTCG AGACCTGTTT 60  
GACCAGCGCCG TCGTCCTGTC CCACTACATC CATAACCTCT CCTCAGAAAT GTTCAGCGAA 120  
TTCGATAAAC GGTATAACCA TGGCCGGGGG TTCATTACCA AGGCCATCAA CAGCTGCCAC 180  
ACTTCTTCCC TTGCCACCCC CGAAGACAAG GAGCAAGCCC AACAGATGAA TCAAAAAGAC 240  
20 TTTCTGAGCC TGATAGTCAG CATATTGCGA TCCTGGAATG AGCCTCTGTA TCATCTGGTC 300  
ACGGAAGTAC GTGGTATGCA AGAAGCCCCG GAGGCTATCC TATCCAAAGC TGTAGAGATT 360  
GAGGAGCAAA CCAAACGGCT TCTAGAGGGC ATGGAGCTGA TAGTCAGCCA GGTCATCCT 420  
GAAACCAAAG AAAATGAGAT CTACCCGTGTC TGGTCGGGAC TTCCATCCCT GCAGATGGCT 480  
GATGAAGAGT CTCGCCTTTC TGCTTATTAT AACCTGCTCC ACTGCCTACG CAGGGATTCA 540  
25 CATAAAATCG ACAATTATCT CAAGCTCCTG AAGTGCCGAA TCATCCACAA CAACAACTGC 600  
TAA;

16K hPRL (Met<sup>-1</sup>Thr<sup>123</sup>) (SEQ ID NO:2)

ATGTTGCCCA TCTGTCCCGG CGGGGCTGCC CGATGCCAGG TGACCCCTTCG AGACCTGTTT 60  
GACCAGCGCCG TCGTCCTGTC CCACTACATC CATAACCTCT CCTCAGAAAT GTTCAGCGAA 120  
30 TTCGATAAAC GGTATAACCA TGGCCGGGGG TTCATTACCA AGGCCATCAA CAGCTCCCAC 180  
ACTTCTTCCC TTGCCACCCC CGAAGACAAG GAGCAAGCCC AACAGATGAA TCAAAAAGAC 240  
TTTCTGAGCC TGATAGTCAG CATATTGCGA TCCTGGAATG AGCCTCTGTA TCATCTGGTC 300  
ACGGAAGTAC GTGGTATGCA AGAAGCCCCG GAGGCTATCC TATCCAAAGC TGTAGAGATT 360  
GAGGAGCAAA CCTAA;

35 16K hPRL (Met<sup>-1</sup>Pro<sup>139</sup>) (SEQ ID NO:3)

ATGTTGCCCA TCTGTCCCGG CGGGGCTGCC CGATGCCAGG TGACCCCTTCG AGACCTGTTT 60

GACCGCGCCG TCGTCCTGTC CCACTACATC CATAACCTCT CCTCAGAAAT GTTCAGCGAA 120  
 TTCGATAAAC GGTATAACCA TG GCCGGGGG TTCATTACCA AGGCCATCAA CAGCTCCCAC 180  
 ACTTCTTCCC TTGCCACCCC CGAAGACAAG GAGCAAGCCC AACAGATGAA TCAAAAAGAC 240  
 TTTCTGAGCC TGATAGTCAG CATATTGCGA TCCTGGAATG AGCCTCTGTA TCATCTGGTC 300  
 5 ACGGAAGTAC GTGGTATGCA AGAAGCCCCG GAGGCTATCC TATCCAAAGC TGTAGAGATT 360  
 GAGGAGCAAA CCAAACGGCT TCTAGAGGGC ATGGAGCTGA TAGTCAGCCA GGTCATCCT 420  
 TGA;

16K hPRL (Met<sup>-1</sup>Pro<sup>142</sup>) (SEQ ID NO:4)

ATGTTGCCCA TCTGTCGGG CGGGGCTGCC CGATGCCAGG TGACCCCTTCG	50
10 AGACCTGTTT GACCGCGCCG TCGTCCTGTC CCACTACATC CATAACCTCT CCTCAGAAAT GTTCAGCGAA TTGATAAAC GGTATAACCA TG GCCGGGGG TTCATTACCA AGGCCATCAA CAGCTCCCAC ACTTCTTCCC TTGCCACCCC CGAAGACAAG GAGCAAGCCC AACAGATGAA TCAAAAAGAC TTTCTGAGCC TGATAGTCAG CATATTGCGA TCCTGGAATG AGCCTCTGTA TCATCTGGTC	100 150 200 250 300
15 ACGGAAGTAC GTGGTATGCA AGAAGCCCCG GAGGCTATCC TATCCAAAGC TGTAGAGATT GAGGAGCAAA CCAAACGGCT TCTAGAGGGC ATGGAGCTGA TAGTCAGCCA GGTCATCCT AGACCCCAA CACCTGAGAT CTACCTGTC	350 400 450 500
TGGTCGGGAC TTCCATCCCT GCAGATGGCT GATGAAGAGT CTCGCCTTTC TGCTTATTAT AACCTGCTCC ACTGCCTACG CAGGGATTCA CATAAAATCG	550
20 ACAATTATCT CAAGCTCCTG AAGTGCCGAA TCATCCACAA CAACAACTGC TAA;	600

hPRL (Met<sup>-1</sup>Cys<sup>199</sup>) antisense (SEQ ID NO:5)

TACAACGGGT AGACAGGGCC GCCCGACGG GCTACGGTCC ACTGGGAAGC TCTGGACAAA	60
CTGGCGCGGC AGCAGGACAG GGTGATGTAG GTATTGGAGA GGAGTCCTTA CAAGTCGCTT	120
25 AAGCTATTTG CCATATGGGT ACCGGCCCCC AAGTAATGGT TCCGGTAGTT GTCGACGGTG	180
TGAAGAAGGG AACGGTGGGG GCTTCTGTT CTCGTTGGG TTGTCTACTT AGTTTTCTG	240
AAAGACTCGG ACTATCAGTC GTATAACGCT AGGACCTTAC TCGGAGACAT AGTAGACCAG	300
TGCCTTCATG CACCATACGT TCTTCGGGGC CTCCGATAGG ATAGGTTTCG ACATCTCTAA	360
CTCCTCGTT GGTTGCCGA AGATCTCCCG TACCTCGACT ATCAGTCGGT CCAAGTAGGA	420
30 CTTTGGTTTC TTTTACTCTA GATGGACAG ACCAGCCCTG AAGGTAGGGA CGTCTACCGA	480
CTACTTCTCA GAGCGAAAG ACGAATAATA TTGGACGAGG TGACGGATGC GTCCCTAAGT	540
GTATTTAGC TGTAAATAGA GTTCGAGGAC TTCACGGCTT AGTAGGTGTT GTTGTGACG	600
ATT;	

16K hPRL (Met<sup>-1</sup>Thr<sup>123</sup>) antisense (SEQ ID NO:6)

35 TACAACGGGT AGACAGGGCC GCCCGACGG GCTACGGTCC ACTGGGAAGC TCTGGACAAA	60
CTGGCGCGGC AGCAGGACAG GGTGATGTAG GTATTGGAGA GGAGTCCTTA CAAGTCGCTT	120
AAGCTATTTG CCATATGGGT ACCGGCCCCC AAGTAATGGT TCCGGTAGTT GTCGAGGGTG	180

TGAAGAAGGG AACGGTGGGG GCTTCTGTT CTCGTTGGG TTGTCTACTT AGTTTTCTG 240  
 AAAGACTCGG ACTATCAGTC GTATAACGCT AGGACCTTAC TCGGAGACAT AGTAGACCAG 300  
 TGCCTTCATG CACCATAACGT TCTCGGGGC CTCCGATAGG ATAGGTTTCG ACATCTCTAA 360  
 CTCCCTCGTTT GGATT;

5 16K hPRL (Met<sup>-1</sup>Pro<sup>139</sup>) antisense (SEQ ID NO:7)  
 TACAACGGGT AGACAGGGCC GCCCCGACGG GCTACGGTCC ACTGGGAAGC TCTGGACAAA 60  
 CTGGCGCGGC AGCAGGACAG GGTGATGTAG GTATTGGAGA GGAGTCTTTA CAAGTCGCTT 120  
 AAGCTATTTG CCATATGGGT ACCGGCCCC AAGTAATGGT TCCGGTAGTT GTCGAGGGTG 180  
 TGAAGAAGGG AACGGTGGGG GCTTCTGTT CTCGTTGGG TTGTCTACTT AGTTTTCTG 240  
 10 AAAGACTCGG ACTATCAGTC GTATAACGCT AGGACCTTAC TCGGAGACAT AGTAGACCAG 300  
 TGCCTTCATG CACCATAACGT TCTCGGGGC CTCCGATAGG ATAGGTTTCG ACATCTCTAA 360  
 CTCCCTCGTTT GGTTTGCCGA AGATCTCCCG TACCTCGACT ATCAGTCGGT CCAAGTAGGA 420  
 ACT;

15 16K hPRL (Met<sup>-1</sup>Pro<sup>142</sup>) (SEQ ID NO:8)  
 TACAACGGGT AGACAGGGCC GCCCCGACGG GCTACGGTCC ACTGGGAAGC 50  
 TCTGGACAAA CTGGCGCGGC AGCAGGACAG GGTGATGTAG GTATTGGAGA 100  
 GGAGTCTTTA CAAGTCGCTT AAGCTATTTG CCATATGGGT ACCGGCCCC 150  
 AAGTAATGGT TCCGGTAGTT GTCGAGGGTG TGAAGAAGGG AACGGTGGGG 200  
 20 GCTTCTGTT CTCGTTGGG TTGTCTACTT AGTTTTCTG AAAGACTCGG 250  
 ACTATCAGTC GTATAACGCT AGGACCTTAC TCGGAGACAT AGTAGACCAG 300  
 TGCCTTCATG CACCATAACGT TCTCGGGGC CTCCGATAGG ATAGGTTTCG 350  
 ACATCTCTAA CTCCTCGTTT GGTTTGCCGA AGATCTCCCG TACCTCGACT 400  
 ATCAGTCGGT CCAAGTAGGA TCTGGGGGTT GTGGACTCTA GATGGGACAG 450  
 25 ACCAGCCCTG AAGGTAGGGA CGTCTACCGA CTACTTCTCA GAGCGGAAAG 500  
 ACGAATAATA TTGGACGGAGG TGACGGATGC GTCCCTAAAGT GTATTTTAGC 550  
 TGTAAATAGA GTTCGAGGAC TTCACGGCTT AGTAGGTGTT GTTGTGACG 600  
 ATT;

hPRL (Met<sup>-1</sup>Cys<sup>199</sup>) (SEQ ID NO:9)  
 30 MetLeuProIleCysProGlyGlyAlaAlaArgCysGlnValThrLeuArgAspLeuPhe  
     -1              5              10              15  
     AspArgAlaValValLeuSerHisTyrIleHisAsnLeuSerSerGluMetPheSerGlu  
     20              25              30              35  
     PheAspLysArgTyrThrHisGlyArgGlyPheIleThrLysAlaIleAsnSerCysHis  
 35     40              45              50              55  
     ThrSerSerLeuAlaThrProGluAspLysGluGlnAlaGlnGlnMetAsnGlnLysAsp  
     60              65              70              75  
     PheLeuSerLeuIleValSerIleLeuArgSerTrpAsnGluProLeuTyrHisLeuVal

80	85	90	95
ThrGluValArgGlyMetGlnGluAlaProGluAlaIleLeuSerLysAlaValGluIle			
100	105	110	115
GluGluGlnThrLysArgLeuLeuGluGlyMetGluLeuIleValSerGlnValHisPro			
5	120	125	130
GluThrLysGluAsnGluIleTyrProValTrpSerGlyLeuProSerLeuGlnMetAla			
140	145	150	155
AspGluGluSerArgLeuSerAlaTyrTyrAsnLeuLeuHisCysLeuArgArgAspSer			
160	165	170	175
10	HisLysIleAspAsnTyrLeuLysLeuLeuLysCysArgIleIleHisAsnAsnAsnCys ;		
180	185	190	195
			199

16K hPRL (Met<sup>-1</sup>Thr<sup>123</sup>) (SEQ ID NO:10)

MetLeuProIleCysProGlyGlyAlaAlaArgCysGlnValThrLeuArgAspLeuPhe			
-1	5	10	15
15	AspArgAlaValValLeuSerHisTyrIleHisAsnLeuSerSerGluMetPheSerGlu		
20	25	30	35
PheAspLysArgTyrThrHisGlyArgGlyPheIleThrLysAlaIleAsnSerSerHis			
40	45	50	55
ThrSerSerLeuAlaThrProGluAspLysGluGlnAlaGlnGlnMetAsnGlnLysAsp			
20	60	65	70
PheLeuSerLeuIleValSerIleLeuArgSerTrpAsnGluProLeuTyrHisLeuVal			
80	85	90	95
ThrGluValArgGlyMetGlnGluAlaProGluAlaIleLeuSerLysAlaValGluIle			
100	105	110	115
25	GluGluGlnThr;		
120	123	,	

16K hPRL (Met<sup>-1</sup>Pro<sup>139</sup>) (SEQ ID NO:11)

MetLeuProIleCysProGlyGlyAlaAlaArgCysGlnValThrLeuArgAspLeuPhe			
-1	5	10	15
30	AspArgAlaValValLeuSerHisTyrIleHisAsnLeuSerSerGluMetPheSerGlu		
20	25	30	35
PheAspLysArgTyrThrHisGlyArgGlyPheIleThrLysAlaIleAsnSerSerHis			
40	45	50	55
ThrSerSerLeuAlaThrProGluAspLysGluGlnAlaGlnGlnMetAsnGlnLysAsp			
35	60	65	70
PheLeuSerLeuIleValSerIleLeuArgSerTrpAsnGluProLeuTyrHisLeuVal			

45

80	85	90	95	
ThrGluValArgGlyMetGlnGluAlaProGluAlaIleLeuSerLysAlaValGluIle				
100	105	110	115	
GluGluGlnThrLysArgLeuLeuGluGlyMetGluLeuIleValSerGlnValHisPro;				
5 120	125	130	135	139

16K hPRL (Met<sup>-1</sup>Pro<sup>142</sup>) (SEQ ID NO:12)

-1	5	10	15	
MetLeuProIleCysProGlyGlyAlaAlaArgCysGlnValThrLeuArgAspLeuPhe				
10 20	25	30	35	
AspArgAlaValValLeuSerHisTyrIleHisAsnLeuSerSerGluMetPheSerGlu				
40	45	50	55	
PheAspLysArgTyrThrHisGlyArgGlyPheIleThrLysAlaIleAsnSerSerHis				
60	65	70	75	
ThrSerSerLeuAlaThrProGluAspLysGluGlnAlaGlnGlnMetAsnGlnLysAsp				
15 80	85	90	95	
PheLeuSerLeuIleValSerIleLeuArgSerTrpAsnGluProLeuTyrHisLeuVal				
100	105	110	115	
ThrgluValArgGlyMetGlnGluAlaProGluAlaIleLeuSerLysAlaValGluIle				
20 120	125	130	135	
GluGluGlnThrLysArgLeuLeuGluGlyMetGluLeuIleValSerGlnValHisPro				
ArgProPro;				
140 142				

hPL (Met<sup>-1</sup>Phe<sup>191</sup>) (SEQ ID NO:13)

25	GCGCACCAGC TGGCCATTGA CACCTACCAAG GAGTTTGAAG AAACCTATAT CCCAAAGGAC	60
CAGAAGTATT CGTTCTGCA TGACTCCCAG ACCTCTTCT GCTTCTCAGA CTCTATTCCG	120	
ACACCCCTCCA ACATGGAGGA AACGCAACAG AAATCCAATC TAGAGCTGCT CCGCATCTCC	180	
CTGCTGCTCA TCGAGTCGTG GCTGGAGCCC GTGCGGTTCC TCAGGAGTAT GTTCGCCAAC	240	
AACCTGGTGT ATGACACCTC GGACAGCGAT GACTATCACC TCCTAAAGGA CCTAGAGGAA	300	
30 GGCATCCAAA CGCTGATGGG GAGGCTGGAA GACGGCAGCC GCCGGACTGG GCAGATCCTC	360	
AAGCAGACCT ACAGCAAGTT TGACACAAAC TCGCACAAACC ATGACGCACT GCTCAAGAAC	420	
TACGGGCTGC TCTACTGCTT CAGGAAGGAC ATGGACAAGG TCGAGACATT CCTGCGCATG	480	
GTGCAGTGCC GCTCTGTGGA GGGCAGCTGT GGCTTCTAG;	540	
	579	

16K hPL (Met<sup>-1</sup>Arg<sup>134</sup>) (SEQ ID NO:14)

	ATGGTCCAAA CCGTCCGTT ATCCAGGCTT TTTGACCACG CTATGCTCCA	50
	AGCCCCATCGC CGGCACCAGC TG GCCATTGA CACCTACCAG GAGTTTGAAG	100
	AAACCTATAT CCCAAAGGAC CAGAAGTATT CGTT CCTGCA TGACTCCCAG	150
5	ACCTCCTTCT CTTTCTCAGA CTCTATTCCG ACACCCCTCCA ACATGGAGGA	200
	AACGCAACAG AAATCCAATC TAGAGCTGCT CGGCATCTCC CTGCTGCTCA	250
	TCGAGTCGTG GCTGGAGCCC GTGCCGGTCC TCAGGAGTAT GTTCGCCAAC	300
	AACCTGGTGT ATGACACCTC GGACAGCGAT GACTATCACC TCCTAAAGGA	350
	CCTAGAGGAA GGCATCCAAA CGCTGATGGG GAGGCTGGAA GACGGCAGCC	400
10	CCCGGACTGG GCAGATCCTC AAGCAGACCT ACAGCAAGTT TGACACAAAC	450
	TCGCACAACC ATGACGCCT GCTCAAGAAC TACGGGCTGC TCTACTGCTT	500
	CAGGAAGGAC ATGGACAAGG TCGAGACATT CCTGCGCATG GTGCAGTGCC	550
	GCTCTGTGGA GGGCAGCTGT GGCTTCTAG;	

hPL (Met<sup>-1</sup>Phe<sup>191</sup>) antisense (SEQ ID NO:15)

15	TAC CAGGTTT GGCAAGGCAA TAGGTCCGAA AAACTGGTGC GATA CGAGGT TCGGGTAGCG 60	
	CGCGTGGTCG ACCGGTAAC GTGGATGGTC CTCAAACCTTC TTTGGATATA GGGTTTCCTG 120	
	GTCTTCATAA GCAAGGACGT ACTGAGGGTC TGGAGGAAGA CGAAGAGTCT GAGATAAGGC 180	
	TGTGGGAGGT TGTACCTCCT TTGCGTTGTC TTTAGGTTAG ATCTCGACGA GGCGTAGAGG 240	
	GACGACGAGT AGCTCAGCAC CGACCTCGGG CACGCCAAGG AGTCCTCATA CAAGCGGTGG 300	
20	TTGGACCACA TACTGTGGAG CCTGTCGCTA CTGATAGTGG AGGATTCCT GGATCTCCTT 360	
	CCGTAGGTTT GCGACTACCC CTCCGACCTT CTGCCGTCGG CGGCCTGACC CGTCTAGGAG 420	
	TTCGTCTGGA TGCGTCAA ACTGTGTTTG AGCGTGTGG TACTGCGTGA CGAGTTCTTG 480	
	ATGCCCGACG AGATGACGAA GTCCTTCCTG TACCTGTTCC AGCTCTGTAA GGACGCGTAC 540	
	CACGTACCGG CGAGACACCT CCCGTCGACA CCGAAGATC;	

16K hPL (Met<sup>-1</sup>Arg<sup>134</sup>) antisense (SEQ ID NO:16)

	TAC CAGGTTT GGCAAGGCAA TAGGTCCGAA AAACTGGTGC GATA CGAGGT	50
	TCGGGTAGCG CGCGTGGTCG ACCGGTAAC GTGGATGGTC CTCAAACCTTC	100
	TTTGGATATA GGGTTTCCTG GTCTTCATAA GCAAGGACGT ACTGAGGGTC	150
	TGGAGGAAGA GAAAGAGTCT GAGATAAGGC TGTGGGAGGT TGTACCTCCT	200
30	TTGCGTTGTC TTTAGGTTAG ATCTCGACGA GGCGTAGAGG GACCGACGAGT	250
	AGCTCAGCAC CGACCTCGGG CACGCCAAGG AGTCCTCATA CAAGCGGTGG	300
	TTGGACCACA TACTGTGGAG CCTGTCGCTA CTGATAGTGG AGGATTCCT	350
	GGATCTCCTT CGGTAGGTTT GCGACTACCC CTCCGACCTT CTGCCGTCGG	400
	GGGCCTGACC CGTCTAGGAG TTCGTCTGGA TGCGTCAA ACTGTGTTTG	450
35	AGCGTGTGG TACTGCGTGA CGAGTTCTTG ATGCCCGACG AGATGACGAA	500
	GTCCTTCCTG TACCTGTTCC AGCTCTGTAA GGACGCGTAC CACGTACCGG	550
	CGAGACACCT CCCGTCGACA CCGAAGATC;	

hPL (Met<sup>-1</sup>Phe<sup>191</sup>) (SEQ ID NO:17)

MetValGlnThrValProLeuSerArgLeuPheAspHisAlaMetLeuGlnAlaHisArg  
 -1 5 10 15  
 AlaHisGlnLeuAlaIleAspThrTyrGlnGluPheGluGluThrTyrIleProLysAsp  
 5 20 25 30 35  
 GlnLysTyrSerPheLeuHisAspSerGlnThrSerPheCysPheSerAspSerIlePro  
 40 45 50 55  
 ThrProSerAsnMetGluGluThrGlnGlnLysSerAsnLeuGluLeuLeuArgIleSer  
 60 65 70 75  
 10 LeuLeuLeuIleGluSerTrpLeuGluProValArgPheLeuArgSerMetPheAlaAsn  
 80 85 90 95  
 AsnLeuValTyrAspThrSerAspSerAspTyrHisLeuLeuLysAspLeuGluGlu  
 100 100 110 115  
 GlyIleGlnThrLeuMetGlyArgLeuGluAspGlySerArgArgThrGlyGlnIleLeu  
 15 120 125 130 135  
 LysGlnThrTyrSerLysPheAspThrAsnSerHisAsnHisAspAlaLeuLeuLysAsn  
 140 145 150 155  
 TyrGlyLeuLeuTyrCysPheArgLysAspMetAspLysValGluThrPheLeuArgMet  
 160 165 170 175  
 20 ValGlnCysArgSerValGluGlySerCysGlyPhe;  
 180 185 190

16K hPL (Met<sup>-1</sup>Arg<sup>134</sup>) (SEQ ID NO:18)

MetValGlnThrValProLeuSerArgLeuPheAspHisAlaMetLeuGlnAlaHisArg  
 -1 5 10 15  
 25 AlaHisGlnLeuAlaIleAspThrTyrGlnGluPheGluGluThrTyrIleProLysAsp  
 20 25 30 35  
 GlnLysTyrSerPheLeuHisAspSerGlnThrSerPheSerAspSerIlePro  
 40 45 50 55  
 ThrProSerAsnMetGluGluThrGlnGlnLysSerAsnLeuGluLeuLeuArgIleSer  
 30 60 65 70 75  
 LeuLeuLeuIleGluSerTrpLeuGluProValArgPheLeuArgSerMetPheAlaAsn  
 80 85 90 95  
 AsnLeuValTyrAspThrSerAspSerAspTyrHisLeuLeuLysAspLeuGluGlu  
 100 105 110 115  
 35 GlyIleGlnThrLeuMetGlyArgLeuGluAspGlySerProArg;  
 120 125 130

hGH (Met<sup>-1</sup>Phe<sup>191</sup>) (SEQ ID NO:19)

ATGTTCCCAA	CCATTCCCTT	ATCCAGGCTT	TTTGACAAACG	CTATGCTCCG	50
CGCCCATCGT	CTGCACCAGC	TGGCCTTGA	CACCTACCAG	GAGTTTGAAG	100
AAGCCTATAT	CCCAAAGGAA	CAGAAGTATT	CATTCTGCA	GAACCCCCAG	150
5	ACCTCCCTCT	GTTTCTCAGA	GTCTATTCCG	ACACCCCTCCA	200
	AACACAACAG	AAATCCAACC	TAGAGCTGCT	CCGCATCTCC	250
	TCCAGTCGTG	GCTGGAGCCC	GTGCAGTTCC	TCAGGAGTGT	300
	AGCCTGGTGT	ACGGCGCCTC	TGACAGCAAC	GTCTATGACC	350
	CCTAGAGGAA	GGCATCCAAA	CGCTGATGGG	GAGGCTGGAA	400
10	CCCGGACTGG	GCAGATCTTC	AAGCAGACCT	ACAGCAAGTT	450
	TCACACAAACG	ATGACGCACT	ACTCAAGAAC	TACGGGCTGC	500
	CAGGAAGGAC	ATGGACAAGG	TCGAGACATT	CCTGCGCATC	550
	GCTCTGTGGA	GGGCAGCTGT	GGCTTCTAG;		

16K hGH (Met<sup>-1</sup>Pro<sup>133</sup>) (SEQ ID NO:20)

ATGTTCCCAA	CCATTCCCTT	ATCCAGGCTT	TTTGACAAACG	CTATGCTCCG	50
CGCCCATCGT	CTGCACCAGC	TGGCCTTGA	CACCTACCAG	GAGTTTGAAG	100
AAGCCTATAT	CCCAAAGGAA	CAGAAGTATT	CATTCTGCA	GAACCCCCAG	150
15	ACCTCCCTCT	CTTTCTCAGA	GTCTATTCCG	ACACCCCTCCA	200
	AACACAACAG	AAATCCAACC	TAGAGCTGCT	CCGCATCTCC	250
	TCCAGTCGTG	GCTGGAGCCC	GTGCAGTTCC	TCAGGAGTGT	300
	AGCCTGGTGT	ACGGCGCCTC	TGACAGCAAC	GTCTATGACC	350
	CCTAGAGGAA	GGCATCCAAA	CGCTGATGGG	GAGGCTGGAA	400
20	CCTAG;				

hGH (Met<sup>-1</sup>Phe<sup>191</sup>) antisense (SEQ ID NO:21)

TACAAGGGTT	GGTAAGGAA	TAGGTCCGAA	AAACTGTTGC	GATACGAGGC	50
25	GCGGGTAGCA	GACGTGGTCG	ACCGGAAACT	GTGGATGGTC	100
	TTCGGATATA	GGGTTCCCTT	GTCTTCATAA	GTAAGGACGT	150
	TGGAGGGAGA	CAAAGAGTCT	CAGATAAGGC	TGTGGGAGGT	200
	TTGTGTTGTC	TTTAGGTTGG	ATCTCGACGA	GGCGTAGAGG	250
30	AGGTCAGCAC	CGACCTCGGG	CACGTCAAGG	AGTCCTCACA	300
	TCGGACCACA	TGCCGCGGAG	ACTGTCGTTG	CAGATACTGG	350
	GGATCTCCTT	CCGTAGGTTT	GCGACTACCC	CTCCGACCTT	400
	GGGCCTGACC	CGTCTAGAAG	TTCGTCTGGA	TGTCGTTCAA	450
	AGTGTGTTG	TACTGCGTGA	TGAGTTCTTG	ATGCCCGACG	500
35	GTCCTTCCTG	TACCTGTTCC	AGCTCTGTAA	GGACGCGTAG	550
	CGAGACACCT	CCCGTCGACA	CCGAAGATC;		

16K hGH (Met<sup>-1</sup>Pro<sup>133</sup>) antisense (SEQ ID NO:22)

TACAAGGGTT	GGTAAGGAA	TAGGTCCGAA	AAACTGTTGC	GATACGAGGC	50
------------	-----------	------------	------------	------------	----

GCGGGTAGCA GACGTGGTCG ACCGGAAACT GTGGATGGTC CTCAAACCTC  
 100  
 TTCCGGATATA GGGTTTCCCT GTCTTCATAA GTAAGGACGT CTTGGGGGTC  
 150  
 TGGAGGGAGA GAAAGAGTCT CAGATAAGGC TGTGGGAGGT TGTCCCTCCT  
 200  
 TTGTGTTGTC TTTAGGTTGG ATCTCGACGA GGCGTAGAGGG GACGACGAGT  
 250  
 5 AGGTCAGCAC CGACCTCGGG CACGTCAAGG AGTCCTACA GAAGCGGTTG  
 300  
 TCGGACCACA TGCCGCGGAG ACTGTCGTTG CAGATACTGG AGGATTCCT  
 350  
 GGATCTCCTT CCGTAGGTTT GCGACTACCC CTCCGACCTT CTACCGTCGG  
 400  
 GGATC ;

hGH (Met<sup>-1</sup>Phe<sup>191</sup>) (SEQ ID NO:23)

10 MetPheProThrIleProLeuSerArgLeuPheAspAsnAlaMetLeuArgAlaHisArg  
 -1 5 10 15  
 LeuHisGlnLeuAlaPheAspThrTyrGlnGluPheGluGluAlaTyrIleProLysGlu  
 20 25 30 35  
 GlnLysTyrSerPheLeuGlnAsnProGlnThrSerLeuCysPheSerGluSerIlePro  
 15 40 45 50 55  
 ThrProSerAsnArgGluGluThrGlnGlnLysSerAsnLeuGluLeuLeuArgIleSer  
 60 65 70 75  
 LeuLeuLeuIleGlnSerTrpLeuGluProValGlnPheLeuArgSerValPheAlaAsn  
 80 85 90 95  
 20 SerLeuValTyrGlyAlaSerAspSerAsnValTyrAspLeuLeuLysAspLeuGluGlu  
 100 105 110 115  
 GlyIleGlnThrLeuMetGlyArgLeuGluAspGlySerProArgThrGlyGlnIlePhe  
 120 125 130 135  
 LysGlnThrTyrSerLysPheAspThrAsnSerHisAsnAspAspAlaLeuLeuLysAsn  
 25 140 145 150 155  
 TyrGlyLeuLeuTyrCysPheArgLysAspMetAspLysValGluThrPheLeuArgIle  
 160 165 170 175  
 ValGlnCysArgSerValGluGlySerCysGlyPhe;  
 180 185 190

30 16K hGH (Met<sup>-1</sup>Pro<sup>133</sup>) (SEQ ID NO:24)

MetPheProThrIleProLeuSerArgLeuPheAspAsnAlaMetLeuArgAlaHisArg  
 -1 5 10 15  
 LeuHisGlnLeuAlaPheAspThrTyrGlnGluPheGluGluAlaTyrIleProLysGlu  
 20 25 30 35  
 35 GlnLysTyrSerPheLeuGlnAsnProGlnThrSerLeuSerPheSerGluSerIlePro  
 40 45 50 55  
 ThrProSerAsnArgGluGluThrGlnGlnLysSerAsnLeuGluLeuLeuArgIleSer

50

60	65	70	75
LeuLeuLeuIleGlnSerTrpLeuGluProValGlnPheLeuArgSerValPheAlaAsn			
80	85	90	95
SerLeuValTyrGlyAlaSerAspSerAsnValTyrAspLeuLeuLysAspLeuGluGlu			
5 100	105	110	115
GlyIleGlnThrLeuMetGlyArgLeuGluAspGlySerPro;			
120	125	130	

**hGH-V (Met<sup>-1</sup>Phe<sup>191</sup>) (SEQ ID NO:25)**

ATGTTCCCAA CCATTCCCTT ATCCAGGCTT TTTGACAAACG CTATGCTCCG 10 CGCCCCGTCGC CTGTACCAGC TGGCATATGA CACCTATCAG GAGTTTGAAG AAGCCTATAT CCTGAAGGAG CAGAAGTATT CATTCCCTGCA GAACCCCCAG ACCTCCCTCT GCTTCTCAGA GTCTATTCCA ACACCTTCCA ACAGGGTGAA AACGCAGCAG AAATCTAACCC TAGAGCTGCT CCGCATTCTCC CTGCTGCTCA TCCAGTCATG GCTGGAGCCC GTGCAGCTCC TCAGGAGCGT CTTCGCCAAC 15 AGCCTGGTGT ATGGCGCCTC GGACAGCAAC GTCTATCGCC ACCTGAAGGA CCTAGAGGAA GCCATCCAAA CGCTGATGTG GAGGCTGGAA GATGGCAGCC CCCGGACTGG GCAGATCTTC AATCAGTCCT ACAGCAAGTT TGACACAAAAA TCGCACAACG ATGACGCACT GCTCAAGAAC TACGGGCTGC TCTACTGCTT CAGGAAGGAC ATGGACAAGG TCGAGACATT CCTGCGCATC GTGCAGTGCC 20 GCTCTGTGGA GGGCAGCTGT GGCTTCTAG;	50 100 150 200 250 300 350 400 450 500 550
---	--

**16K hGH-V (Met<sup>-1</sup>Arg<sup>134</sup>) (SEQ ID NO:26)**

ATGTTCCCAA CCATTCCCTT ATCCAGGCTT TTTGACAAACG CTATGCTCCG 25 CGCCCCGTCGC CTGTACCAGC TGGCATATGA CACCTATCAG GAGTTTGAAG AAGCCTATAT CCTGAAGGAG CAGAAGTATT CATTCCCTGCA GAACCCCCAG ACCTCCCTCT GCTTCTCAGA GTCTATTCCA ACACCTTCCA ACAGGGTGAA AACGCAGCAG AAATCTAACCC TAGAGCTGCT CCGCATTCTCC CTGCTGCTCA TCCAGTCATG GCTGGAGCCC GTGCAGCTCC TCAGGAGCGT CTTCGCCAAC AGCCTGGTGT ATGGCGCCTC GGACAGCAAC GTCTATCGCC ACCTGAAGGA CCTAGAGGAA GCCATCCAAA CGCTGATGTG GAGGCTGGAA GATGGCAGCC 30 CCCGGACTGG GCAGATCTTC AATCAGTCCT ACAGCAAGTT TGACACAAAAA TCGCACAACG ATGACGCACT GCTCAAGAAC TACGGGCTGC TCTACTGCTT CAGGAAGGAC ATGGACAAGG TCGAGACATT CCTGCGCATC GTGCAGTGCC GCTCTGTGGA GGGCAGCTGT GGCTTCTAG;	50 100 150 200 250 300 350 400 450 500 550
--	--

**hGH-V (Met<sup>-1</sup>Phe<sup>191</sup>) antisense (SEQ ID NO:27)**

35 TACAAGGGTT GGTAAGGGAA TAGGTCCGAA AAACTGTTGC GATACGAGGC GCAGGGCAGCG GACATGGTCG ACCGTATACT GTGGATAGTC CTCAAACCTTC TTCGGATATA GGACTTCCTC GTCTTCATAA GTAAGGACGT CTTGGGGGTC	50 100 150
---	------------------

	TTGGAGGGAGA CGAACAGTCT CAGATAAGGT TGTGGAAGGT TGTCCCAC	200
	TTGCGTCGTC TTTAGATTGG ATCTCGACGA GGCCTAGAGG GACGACGAGT	250
	AGGTCAGTAC CGACCTCGGG CACGTCGAGG AGTCCTCGCA GAAGCGGTTG	300
	TCGGACCACA TACCGCGGAG CCTGTCGTTG CAGATAGCGG TGGACTTCCT	350
5	GGATCTCCTT CCGTAGGTTT GCGACTACAC CTCCGACCTT CTACCGTCGG	400
	GGGCCTGACC CGTCTAGAAG TTAGTCAGGA TGTCGTTCAA ACTGTGTTTT	450
	AGCGTGTGTC TACTGCGTGA CGAGTTCTTG ATGCCGACG AGATGACGAA	500
	GTCCTTCCTG TACCTGTTCC AGCTCTGTAA GGACGCGTAG CACGTCACGG	550
	CGAGACACCT CCCGTCGACA CCGAAGATC;	
10	<b>16K hGH-V (Met<sup>-1</sup>Arg<sup>134</sup>) antisense (SEQ ID NO:28)</b>	
	TACAAGGGTT GGTAAGGGAA TAGGTCCGAA AAACGTGTTGC GATACGAGGC	50
	GCAGGGCAGCG GACATGGTCG ACCGTATACT GTGGATAGTC CTCAAACTTC	100
	TTCGGATATA GGACTTCCTC GTCTTCATAA GTAAGGACGT CTTGGGGGTC	150
	TGGAGGGAGA CGAACAGTCT CAGATAAGGT TGTGGAAGGT TGTCCCAC	200
15	TTGCGTCGTC TTTAGATTGG ATCTCGACGA GGCCTAGAGG GACGACGAGT	250
	AGGTCAGTAC CGACCTCGGG CACGTCGAGG AGTCCTCGCA GAAGCGGTTG	300
	TCGGACCACA TACCGCGGAG CCTGTCGTTG CAGATAGCGG TGGACTTCCT	350
	GGATCTCCTT CCGTAGGTTT GCGACTACAC CTCCGACCTT CTACCGTCGG	400
	GGGCCTGACC CGTCTAGAAG TTAGTCAGGA TGTCGTTCAA ACTGTGTTTT	450
20	AGCGTGTGTC TACTGCGTGA CGAGTTCTTG ATGCCGACG AGATGACGAA	500
	GTCCTTCCTG TACCTGTTCC AGCTCTGTAA GGACGCGTAG CACGTCACGG	550
	CGAGACACCT CCCGTCGACA CCGAAGATC;	

	<b>hGH-V (Met<sup>-1</sup>Phe<sup>191</sup>) (SEQ ID NO:29)</b>	
	Met Phe Pro Thr Ile Pro Leu Ser Arg Leu Phe Asp Asn Ala Met Leu Arg Ala Arg Arg	
25	-1 5 10 15	
	Leu Tyr Gln Leu Ala Tyr Asp Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Leu Lys Glu	
	20 25 30 35	
	Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro	
	40 45 50 55	
30	Thr Pro Ser Asn Arg Val Lys Thr Gln Gln Lys Ser Asn Leu Glu Leu Leu Arg Ile Ser	
	60 65 70 75	
	Leu Leu Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Leu Leu Arg Ser Val Phe Ala Asn	
	80 85 90 95	
	Ser Leu Val Tyr Gly Ala Ser Asp Ser Asn Val Tyr Arg His Leu Lys Asp Leu Glu Glu	
35	100 105 110 115	
	Gly Ile Gln Thr Leu Met Trp Arg Leu Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile Phe	
	120 125 130 135	
	Asn Gln Ser Tyr Ser Lys Phe Asp Thr Lys Ser His Asn Asp Asp Ala Leu Leu Lys Asn	
	140 145 150 155	

TyrGlyLeuLeuTyrCysPheArgLysAspMetAspLysValGluThrPheLeuArgIle  
160 165 170 175  
ValGlnCysArgSerValGluGlySerCysGlyPhe;  
180 185 190

5 16K hGH-V (Met<sup>-1</sup>Arg<sup>134</sup>) (SEQ ID NO:30)  
MetPheProThrIleProLeuSerArgLeuPheAspAsnAlaMetLeuArgAlaArgArg  
-1 5 10 15  
LeuTyrGlnLeuAlaTyrAspThrTyrGlnGluPheGluGluAlaTyrIleLeuLysGlu  
20 25 30 35  
10 GlnLysTyrSerPheLeuGlnAsnProGlnThrSerLeuCysPheSerGluSerIlePro  
40 45 50 55  
ThrProSerAsnArgValLysThrGlnGlnLysSerAsnLeuGluLeuLeuArgIleSer  
60 65 70 75  
LeuLeuLeuIleGlnSerTrpLeuGluProValGlnLeuLeuArgSerValPheAlaAsn  
15 80 85 90 95  
SerLeuValTyrGlyAlaSerAspSerAsnValTyrArgHisLeuLysAspLeuGluGlu  
100 105 110 115  
GlyIleGlnThrLeuMetTrpArgLeuGluAspGlySerProArg.  
120 125 130

20 The sequences of the 16K N-terminal fragments were obtained by mutagenesis of the sequences of the intact hormones. Mutagenesis was performed using the oligonucleotide-directed mutagenesis kit purchased from either Amersham International (Buckinghamshire, UK) or Boehringer  
25 Mannheim (Mannheim, Germany). Oligonucleotides were purchased from Eurogentec S.A. (Seraing, Belgium).

The coding sequences were inserted into expression vector pT7L according to *J. Mol. Biol.*, 89:113-130 (1986). Plasmid constructions were made following standard techniques described in *Molecular Cloning, A Laboratory Manual*, Eds. Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1992). Restriction enzymes and ligase were obtained from Eurogentec S.A. (Seraing, Belgium), Bethesda Research Laboratories (Gaithersburg, MD) or  
30 35 Boehringer Mannheim (Mannheim, Germany).

Expression of the proteins was performed in *E. coli*

BL21(DE3). For purifications, Sephadex G100 or G75 gels (Pharmacia, Uppsala, Sweden) packed in C26 columns (2.6 x-100 cm, Pharmacia) and anion exchange MonoQ, Hitrap Q and Phenyl sepharose 6 fast flow columns (HR 10/10, Pharmacia) mounted on a FPLC system (Pharmacia) were used. Elution profiles were monitored by recording the absorbance at 280 nm.

5                   **Example 4. Production of Human Prolactin**

This example describes the procedure for preparation of intact full-length (23K) human prolactin.

10                  The coding region for human prolactin (hPRL) minus the signal peptide was inserted into plasmid pT7L according to Biotech. *Applied Biochem.*, 12:436 (1990). An initiation codon ATG was added to the 5' end of the coding sequence of the hPRL deoxyribonucleotide sequence (denoted hPRL ( $\text{Met}^{-1}\text{Cys}^{199}$ )) (SEQ ID NO:1) and inserted into the pT7L *E. coli* expression vector. 15 This plasmid was called pT7L-hPRL.

A 100 ml culture of *E. coli* BL21(DE3) carrying the pT7L-hPRL plasmid was grown overnight at 37°C in LB medium-ampicillin 100 µg/ml. Twenty ml of this culture were 20 used to inoculate 1 liter of LB medium-ampicillin 100 µg/ml. When the O.D. (600 nm) reached 0.9, 1 mM isopropyl β-D thiogalactopyranoside (IPTG) was added to the culture. The induced culture was grown for an additional 4 hour period. Cells were collected by centrifugation at 5000 g for 15 min 25 (4°C) and resuspended in 50 mM Tris-HCl, 0.5 mM EDTA, (T<sub>50</sub>E<sub>0.5</sub>), 0.1 mM PMSF, pH 8. Cells were broken in a cell disrupter. After centrifugation of cell lysates (12,000g, 15 min, 4°C), hPRL was found in the insoluble fraction (inclusion bodies).

The inclusion bodies recovered in the pellet were 30 washed 2 times in 250 ml of T<sub>50</sub>E<sub>0.5</sub>, 0.1 mM PMSF, pH 8. Inclusions bodies were solubilized in 200 mM phosphate buffer (pH 7) containing 8 M of deionized urea, 1% β-mercaptoethanol (β-ME), 0.5 mM PMSF at a concentration of 100 µg of protein per ml of buffer. The protein solution was heated at 55°C for 35 5 min and then incubated overnight at room temperature. The renaturation was performed by a continued dialysis (72 hours) against 500 volumes of 50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5.

The renatured protein was concentrated to 2 mg/ml and loaded on a Sephadex G100 column (50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5). Fractions containing hPRL were pooled, dialyzed against 50 volumes of 20 mM Tris-HCl, pH 8 and loaded on a HitrapQ anion exchange column. Chromatography was performed in 20 mM Tris-HCl, pH 8 and hPRL was eluted within a gradient 0 to 1 M NaCl. Fractions containing purified hPRL were pooled, dialyzed against 20 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5 and lyophilized. The protein was purified to 95% homogeneity.

10           Example 5. Production of the 16K N-terminal Fragment of Human Prolactin

This example describes the construction of plasmids coding for 3 different forms of 16K hPRL.

15           The 16K N-terminal fragment of human prolactin was produced by three different methods. The DNA sequences coding for the three different forms of 16K hPRL have been obtained by mutating the DNA of hPRL (cloned in the laboratory) (SEQ ID NO:1). The three different forms of 16K hPRL are called 16K hPRL (stop 124), 16K hPRL (stop 140) and 16K hPRL (IgA). All 20 of the forms exhibit the same antiangiogenic activity.

25           To generate 16K hPRL (stop 124), two mutations were introduced in the hPRL coding sequence (16K hPRL (Met<sup>-1</sup>Thr<sup>123</sup>)) (SEQ ID NO:2). The Lys124 codon (AAA) was mutated to a stop codon (TAA); the Cys58 (TGC) into a Ser (TCC). The resulting plasmid was called pT7L-16K hPRL (stop 124). It encodes a protein called 16K hPRL (stop 124) which is composed of the 123 N-terminal amino acids of hPRL with the substitution of a Ser at position 58 instead of a Cys (SEQ ID NO:10).

30           To generate 16K hPRL (stop 140), two mutations were introduced in the hPRL cDNA (16K hPRL (Met<sup>-1</sup>Pro<sup>139</sup>)) (SEQ ID NO:3). The Glu 140 codon (GAA) was mutated in a stop codon (TGA); the Cys 58 (TGC) into a Ser (TCC). The resulting plasmid was called pT7L-16K hPRL (stop 140). It encodes a 35 protein called 16K hPRL (stop 140) which is composed of the 139 N-terminal amino acids of hPRL with the substitution of a Ser at position 58 instead of a Cys (SEQ ID NO:11).

To obtain 16K hPRL (IgA) mutant, Cys 58 codon (TGC) was mutated to Ser codon (TCC) and the nucleotide sequence CCTGAAACCA AAGAAAAT (SEQ NO:31) coding for amino acids 139-144 ProGluThrLysGluAsn (SEQ ID NO:32) in hPRL was replaced by the 5 nucleotide sequence CCTAGACCCC CAACACCT (SEQ ID NO:33) coding for the specific cleavage site of the IgA protease (ProArgProProThrPro) (SEQ ID NO:34). The resulting plasmid was called pT7L-hPRL (IgA). It encodes a protein called hPRL (IgA) which differs from hPRL at position 58 (Ser) and 140-144 10 (IgA specific cleavage site) (16K hPRL (Met<sup>-1</sup>Pro<sup>142</sup>)).

After cleavage of hPRL (IgA) by the IgA protease (between Pro 142 and Thr 143), the N-terminal fragment denoted 16K hPRL (IgA) is released. The latter was composed of the 15 142 N-terminal residues of hPRL with the substitution of a Ser at position 58 and Arg-Pro-Pro at position 140-142 (SEQ ID NO:12).

To produce and purify 16K hPRL (stop124) a 100 ml culture of *E. coli* BL21(DE3) carrying the pT7L-16K 20 hPRL (stop 124) plasmid was grown overnight at 37°C in LB medium-ampicillin 100 µg/ml. Twenty ml of this culture were used to inoculate 1 liter of LB medium-ampicillin 100 µg/ml. When the O.D. (600 nm) reached at 0.9, 1 mM isopropyl β-D thiogalactopyranoside (IPTG) was added to the culture. The induced culture was grown for an additional 4 hour period. 25 Cells were collected by centrifugation at 5000 g for 15 min (4°C) and resuspended in 50 mM Tris-HCl, 0.5 mM EDTA, (T<sub>50</sub>E<sub>0.5</sub>), 0.1 mM PMSF, pH 8. Cells were broken in a French Pressure cell Press (American Instrument Co.) at 13500 psi. After centrifugation of cell lysates (12,000g, 15 min, 4°C) , 16K 30 hPRL (stop124) was found in both soluble and insoluble fractions (inclusion bodies).

The inclusion bodies were recovered in the pellet and washed 2 times in 250 ml of T<sub>50</sub>E<sub>0.5</sub>, 0. 1 mM PMSF, pH 8. Inclusions bodies were solubilized in 200 mM phosphate buffer 35 (pH 7) containing 8 M of deionized urea, 1% O-ME, 0.5 mM PMSF at a concentration of 100 µg of protein per ml of buffer. The protein solution was heated at 55°C for 5 min and then incubated overnight at room temperature. The renaturation was

performed by a continued dialysis (72 hours) against 500 volumes of 50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5.

The renatured protein was finally dialyzed against of 20 mM Tris-HCl, pH 8. After centrifugation at 5000 g to remove any precipitate, the supernatant was loaded on a MonoQ anion exchange column. Chromatography was performed in 20 mM Tris-HCl, pH 8 and 16K hPRL (stop124) was eluted within a gradient 0 to 1 M NaCl. Fractions containing 16K hPRL (stop124) were pooled and dialyzed against 50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5. After centrifugation, supernatant was loaded on a Sephadex G75 column (50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5). Fractions containing 16K hPRL (stop124) were pooled, dialyzed against 20 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5 and lyophilized.

Purification of the soluble fraction of 16K hPRL (stop124) was performed. The supernatant obtained after centrifugation of cell lysate was fractionated with ammonium sulfate (4°C). The precipitate obtained by the 10 to 40% fractionation was collected by centrifugation. The protein pellet corresponding to 1 liter of culture was resuspended in 50 ml of 50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5 and dialyzed overnight (4°C) against the same buffer containing 0.5 mM EDTA, 0.1 mM PMSF and 1 mM NaN<sub>3</sub>. After centrifugation, the 16K hPRL (stop124) was dialyzed against 50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5. The protein was then concentrated to 2 mg/ml. The 16K hPRL (stop124) was denatured in 200 mM phosphate buffer, pH 7, containing 8 M of deionized urea, 1% β-ME, 0.5 mM PMSF.

The solution was heated at 55°C for 5 min and then incubated overnight at room temperature. The denatured proteins were loaded on a Sephadex G100 column equilibrated in buffer containing 6 M deionized urea, 50 mM NH<sub>4</sub>HCO<sub>3</sub>, 5 mM β-ME, 0.5 mM PMSF, pH 7.5. Fractions containing 16K hPRL (stop124) were pooled, and renatured by continued dialysis (72 hours) against 500 volumes of 50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5.

Renatured proteins were finally dialyzed against 20 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5 and lyophilized. Degree of purity was between 90 and 95%.

The molecular mass of the 16K hPRL (stop124) was calculated from its amino acid sequence to be 13.9 kD.

To produce 16K hPRL (stop 140) an *E. coli* BL21(DE3) culture (100 ml) carrying the plasmid pT7L-16K hPRL (stop-140) was grown overnight at 37°C into TB medium-ampicillin 100 µg/ml. Twenty ml of this culture was used to inoculate 1 liter of TB medium-ampicillin 100 µg/ml. When the O.D. (600 nm) reached at 0.9, 1 mM isopropyl β-D thiogalactopyranoside (IPTG) was added to the culture. The induced culture was grown for an additional 4 hour period.

Cells were collected by centrifugation at 5000 g for 15 min (4°C) and resuspended in 50 mM Tris-HCl, 0.5 mM EDTA, ( $T_{50}E_{0.5}$ ), 0.1 mM PMSF, pH 8. Cells were broken in a French Pressure Cell Press (American Instrument Co.) at 13500 psi. After centrifugation of cell lysates (12,000g, 15 min, 4°C), 16K hPRL (stop140) was found in the insoluble fraction (inclusion bodies). The latter were solubilized in 20 mM ethanolamine-HCl, pH 9, containing 8 M deionized urea, 1% β-ME, 0.5 mM PMSF at a ratio of 100 µg of protein per ml of buffer.

The solution was heated at 55°C for 5 min and then incubated overnight at RT. The denatured proteins were dialyzed against 20 mM ethanolamine-HCl, pH 9, 6 M deionized urea. After centrifugation (5000g; 15 min.), the proteins were applied for purification onto an anion exchange Hitrap Q column. Chromatography was performed in 20 mM ethanolamine-HCl, pH 9, 6 M deionized urea and 16K hPRL (stop140) was eluted within a gradient 0 to 1 M NaCl.

Fractions containing 16K hPRL (stop 140) were pooled, diluted with 20 mM ethanolamine-HCl, pH 9, 6 M deionized urea to a concentration of 0.1 mg/ml. For the renaturation, a continued dialysis was performed within a bath of 10 volumes of 20 mM ethanolamine-HCl, pH 9, 6 M deionized urea whose the urea was progressively removed by buffer exchange against 500 volumes of 20 mM ethanolamine, pH 9 for 72 hours. The renatured proteins were then dialyzed against 50 mM NH<sub>4</sub>HCO<sub>3</sub>, PH 7.5, 0.1 M NaCl. After concentration until 2 mg/ml, the proteins were loaded a Sephadex G100 column (50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5, 0.1 M NaCl). Fractions containing the 16K hPRL (stop 140) were pooled, dialyzed against 20 mM NH<sub>4</sub>HCO<sub>3</sub>, pH

7.5 and lyophilized.

The molecular mass of the 16K hPRL (IgA) calculated from its amino acid sequence was 15.8 kD.

To produce 16K hPRL (IgA) an *E. coli* BL21(DE3) culture (100 ml) carrying the plasmid pT7L-hPRL (IgA) was grown overnight at 37°C in LB medium-ampicillin 100 µg/ml. Twenty ml of this culture was used to inoculate 1 liter of LB medium-ampicillin 100 µg/ml. When the O.D. (600 nm) reached at 0.9, 1 mM isopropyl β-D thiogalactopyranoside (IPTG) was added to the culture. The induced culture was grown for an additional 4 hour period. Cells were collected by centrifugation at 5000 g for 15 min (4°C) and resuspended in 50 mM Tris-HCl, 0.5 mM EDTA, ( $T_{50}E_{0.5}$ ), 0.1 mM PMSF, pH 8. Cells were broken in a cell disrupter. After centrifugation of cell lysates (12,000g, 15 min, 4°C), 23K hPRL (IgA) was found in the insoluble fraction (inclusion bodies).

The inclusion bodies recovered in the pellet were washed 2 times in 250 ml of  $T_{50}E_{0.5}$  0.1 mM PMSF, pH 8. Inclusions bodies were solubilized in 200 mM phosphate buffer (pH 7) containing 8 M of deionized urea, 1% β-ME, 0.5 mM PMSF at a concentration of 100 µg of protein per ml of buffer. The protein solution was heated at 55°C for 5 min and then incubated overnight at room temperature. The renaturation was performed by a continued dialysis (72 hours) against 500 volumes of 50 mM  $NH_4HCO_3$ , pH 7.5. After renaturation, the 23K hPRL(IgA) was dialyzed against 50 mM  $NH_4HCO_3$ , pH 7.5. For the digestion by IgA protease, the protein solution was concentrated to 1 mg/ml. The proteins were incubated 24 hours (25°C) with 0.05% (w/w) of IgA protease (Boehringer Mannheim, Mannheim, Germany). The cleaved proteins were incubated for one hour with 1% β-ME before loading on a Sephadex G100 column (50 mM  $NH_4HCO_3$ , pH 7.5, 100 mM NaCl, 5 mM β-ME). Fractions containing 16K hPRL(IgA) were pooled, dialyzed against 20 mM Tris-HCl, pH 8 and loaded on a HitrapQ anion exchange column. Chromatography was performed in 20 mM Tris-HCl, pH 8 and 16K hPRL(IgA) was eluted within a gradient 0 to 1 M NaCl. Fractions containing purified 16K hPRL (IgA) were pooled, dialyzed against 20 mM  $NH_4HCO_3$ , pH 7.5 and lyophilized.

Proteins were purified to 95% homogeneity.

The molecular mass of the 16K hPRL (IgA) calculated from its amino acid sequence was determined to be 16.1 kD.

5                   Example 6. Production of Intact Human Growth Hormone

This example describes a procedure for preparation of intact human growth hormone (hGH(Met<sup>-1</sup>Phe<sup>191</sup>)) (SEQ ID NO:23).

10                  The coding region for human growth hormone (hGH) minus the signal peptide was inserted into plasmid pT7L. An initiation codon ATG was added to the 5' end of the coding sequence of the hGH (*Science*, 205:602 (1979) (SEQ ID NO:19), and inserted into the pT7L *E. coli* expression vector. This plasmid was called pT7L-hGH.

15                  A 100 ml culture of *E. coli* BL21(DE3) carrying the pT7L-hGH plasmid was grown at 37°C in LB medium-ampicillin 100 µg/ml. Twenty ml of this culture were used to inoculate 1 liter of LB medium-ampicillin 100 µg/ml. When the O.D. (600 nm) reached at 0.9, 1 mM isopropyl β-D thiogalactopyranoside (IPTG) was added to the culture. The induced culture was grown for an additional 4 hour period. Cells were collected by centrifugation at 5000 g for 15 min (4°C) and resuspended in 50 mM Tris-HCl, 0.5 mM EDTA, (T<sub>50</sub>E<sub>0.5</sub>), 0.1 mM PMSF, pH 8. Cells were broken in a cell disrupter. After centrifugation 20 of cell lysates (12,000g, 15 min, 4°C), hGH was found in insoluble fractions (inclusion bodies).

25                  The inclusion bodies recovered in the pellet were washed 2 times in 250 ml of T<sub>50</sub>E<sub>0.5</sub> 0.1 mM PMSF, pH 8 and stored at -20°C. Inclusion bodies were solubilized in 20 mM ethanolamine, pH 9 containing 8 M of deionized urea, 1% β-ME, 0.5 mM PMSF at a concentration of 100 µg of protein per ml of buffer. The protein solution was heated at 55°C for 5 min and then incubated overnight at room temperature. The renaturation was performed by a continued dialysis (72 hours) 30 against 500 volumes of 20 mM ethanolamine, pH 9.

35                  The renatured protein was loaded on a HitrapQ anion exchange column. Chromatography was performed in 20 mM ethanolamine, pH 9 and hGH was eluted within a gradient 0 to 1

M NaCl. Fractions containing hGH were pooled, concentrated to 2 mg/ml and loaded on a Sephadex G100 column (50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5). Fractions containing purified hGH were pooled, dialyzed against 20 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5 and lyophilized. proteins were 5 purified to 90% homogeneity.

Example 7. Purification of the 16K N-terminal Fragment of Human Growth Hormone

This example describes the procedure for preparation 10 of the 16K N-terminal fragment of human growth hormone (16K hGH (Met<sup>-1</sup>Pro<sup>133</sup>)) (SEQ ID 24).

Two mutations were introduced in the hGH coding sequence. Cys 53 (TGT) was mutated to Ser (TCT) and Arg 134 (CGG) was mutated to a stop codon (TAG) (SEQ ID NO:20). The 15 mutated cDNA was reinserted into the pT7L expression vector and called pT7L-16K hGH.

A *E. coli* BL21(DE3) culture (100 ml) carrying the plasmid pT7L-16K hGH was grown overnight at 37°C into TB medium-ampicillin 100 µg/ml. Twenty ml of this culture was 20 used to inoculate 1 liter of TB medium-ampicillin 100 µg/ml. When the O.D.<sub>600</sub> reached at 0.6, 1 mM isopropyl β-D thiogalactopyranoside (IPTG) was added to the culture. The induced culture was grown for an additional 4 hour period. Cells were collected by centrifugation at 5000 g for 15 min 25 (4°C) and resuspended in 50 mM Tris-HCl, 0.5 mM EDTA, (T<sub>50</sub>E<sub>0.5</sub>), 0.1 mM PMSF, pH 8.

16K hGH was produced as an insoluble form (inclusion bodies). The latter were solubilized in 20 mM ethanolamine-HCl, pH 9, containing 8 M deionized urea, 1% β-ME, 0.5 mM PMSF 30 (Buffer A) at a ratio of 100 µg of protein per ml of buffer. The solution was heated at 55°C for 5 min and then incubated overnight at RT. The denatured proteins were dialyzed against 20 mM ethanolamine-HCl, pH 9, 6 M deionized urea. After 35 centrifugation (5000g; 15 min.), the proteins were applied for purification onto an anion exchange Hitrap Q column. Chromatography was performed in 20 mM ethanolamine-HCl, pH 9, 6 M deionized urea and 16K hGH was eluted within a gradient 0 to 1 M NaCl. Fractions containing 16K hGH were pooled,

diluted with 20 mM ethanolamine-HCl, pH 9, 6 M deionized urea to a concentration of 0.1 mg/ml.

The renaturation was performed by a continued dialysis for 72 hours. For the renaturation, a continued 5 dialysis was performed within a bath of 10 volumes of 20 mM ethanolamine-HCl, pH 9, 6 M deionized urea. The urea was progressively removed by buffer exchange against 500 volumes of 20 mM ethanolamine, pH 9 for 72 hours. The renatured 10 proteins were then dialyzed against 50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5, 0.1 M NaCl. After concentration to 2 mg/ml, the proteins were loaded a Sephadex G100 column (50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5, 0.1 M NaCl). Fractions containing the 16K hGH were pooled, dialyzed against 20 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5 and lyophilized.

The molecular mass of the 16K hGH calculated from 15 its amino acid sequence was determined to be 15.5 kD. Proteins were purified to 95% homogeneity.

Example 8. Production of Intact Human Growth Hormone Variant hGH-V

This example describes a procedure for preparation 20 of intact human growth hormone variant hGH-V (hGH-V(Met<sup>1</sup>Phe<sup>131</sup>)) (SEQ ID NO:29).

The coding region for hGH-V minus the signal peptide was inserted into a plasmid. The coding sequence for hGH-V 25 was cloned by screening a placental cDNA library (Clonetech, HL 1008). An initiation codon ATG was added to the 5' end of the coding sequence of the hGH-V (SEQ ID NO:25) and inserted into the pT7L *E. coli* expression vector. This plasmid was called pT7L-hGH-V.

A 100 ml culture of *E. coli* BL21(DE3) carrying the 30 pT7L-hGH-V plasmid was grown overnight at 37°C in LB medium-ampicillin 100 µg/ml. Twenty ml of this culture were used to inoculate 1 liter of LB medium-ampicillin 100 µg/ml. When the O.D. (600 nm) reached at 0.9, 1 mM isopropyl β-D 35 thiogalactopyranoside (IPTG) was added to the culture. The induced culture was grown for an additional 4 hour period. Cells were collected by centrifugation at 5000 g for 15 min (4°C) and resuspended in 50 mM Tris-HCl, 0.5 mM EDTA, (T<sub>50</sub>E<sub>0.5</sub>),

0.1 mM PMSF, pH 8. Cells were broken in a cell disrupter. After centrifugation of cell lysates (12,000g, 15 min, 4°C), hGH-V was found in the insoluble fraction (inclusion bodies).

The inclusion bodies recovered in the pellet were  
5 washed 2 times in 250 ml of T<sub>5</sub>O<sub>E0.5</sub> 0.1 mM PMSF, pH 8. Inclusion bodies were solubilized in 20 mM ethanolamine (pH 10) containing 8 M of deionized urea, 1% β-ME, 0.5 mM PMSF at a concentration of 100 µg of protein per ml of buffer. The protein solution was heated at 55°C for 5 min and then  
10 incubated overnight at room temperature. The renaturation was performed by a continued dialysis (72 hours) against 500 volumes of 20 mM ethanolamine (pH 10).

The renatured protein was loaded on a HitrapQ anion exchange column. Chromatography was performed in 20 mM ethanolamine, pH 10 and hGH-V was eluted within a gradient of 0 to 1 M NaCl. Fractions containing hGH-V were pooled, concentrated to 2 mg/ml and loaded on a Sephadex G100 column (50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 9). Fractions containing purified hGH-V were pooled, dialyzed against 20 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 9 and  
20 lyophilized. Proteins were purified to 95% homogeneity.

Example 9. Purification of the 16K N-terminal Fragment of Human Growth Hormone Variant hGH-V

This example describes the procedure for preparation of the 16K N-terminal fragment of human growth hormone variant hGH-V (16K hGH-V (Met<sup>-1</sup>Arg<sup>134</sup>)) (SEQ ID NO:30).

For production of 16K hGH-V, a natural cleavage site specific to thrombin is present at position Pro133-Arg134. Cleavage occurs after arginine 134. Human GH-V was produced and inclusion bodies denatured and renatured as described in Example 8. hGH-V was concentrated to 1 mg/ml and then enzymatically cleaved with thrombin (0.3%, 25°C, overnight, Sigma).

Purification was performed. The cleaved hGH-V was denatured in 20 mM ethanolamine-HCl, pH 9, 6 M deionized urea, 1% β-ME, 0.5 mM PMSF. After centrifugation (5000g; 15 min.), the proteins were applied for purification onto a anion exchange Hitrap Q column. Chromatography was performed in 20

5 mM ethanolamine-HCl, pH 9, 6 M deionized urea, 5 mM  $\beta$ -ME and 16K hGH-V was eluted within a gradient 0 to 1 M NaCl. Fractions containing 16K hGH-V were pooled, diluted with 20 mM ethanolamine-HCl, pH 9, 6 M deionized urea to a concentration 5 of 0.1 mg/ml.

The renaturation was performed by a continued dialysis for 72 hours. In order to remove the urea carefully, a first dialysis against 10 volumes of 20 mM ethanolamine-HCl, pH 9, 6 M deionized urea was performed. 10 Second, the urea from this bath was removed by a continued dialysis against 500 volumes of 20 mM ethanolamine, pH 9. For the renaturation, a continued dialysis was performed within a bath of 10 volumes of 20 mM ethanolamine-HCl, pH 9, 6 M deionized urea whose the urea was progressively removed by 15 buffer exchange against 500 volumes of 20 mM ethanolamine, pH 9 for 72 hours. The renatured proteins were then dialyzed against 50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 9, 0.1 M NaCl. After concentration to 2 mg/ml, the proteins were loaded a Sephadex G100 column (50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 9, 0.1 M NaCl). Fractions containing the 20 16K hGH-V were pooled, dialyzed against 20 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 9 and lyophilized.

The molecular mass of the 16K hGH-V calculated from its amino acid sequence was determined to be 15.7 kD.

Example 10. Production of Intact Human Placental  
25 Lactogen

This example describes a procedure for preparation of intact human placental lactogen (hPL(Met<sup>-1</sup>Phe<sup>131</sup>)) (SEQ ID NO:17).

The coding sequence of the hPL was cloned using 30 RT-PCR experiments performed on syncytiotrophoblastic cells. An initiation codon ATG was added to the 5' end of the coding sequence of the hPL (SEQ ID NO:13) and inserted into the pT7L E. coli expression vector. This recombinant plasmid was called pT7L-hPL.

35 A 100 ml culture of E. coli BL21(DE3) carrying the pT7L-hPL plasmid was grown overnight at 37°C in LB medium-ampicillin 100  $\mu$ g/ml. Twenty ml of this culture were

- used to inoculate 1 liter of LB medium-ampicillin 100  $\mu$ g/ml. When the O.D. (600 nm) reached at 0.9, 1 mM isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) was added to the culture. The induced culture was grown for an additional 4 hour period.
- 5 Cells were collected by centrifugation at 5000 g for 15 min (4°C) and resuspended in 50 mM Tris-HCl, 0.5 mM EDTA, ( $T_{50}E_{0.5}$ ), 0.1 mM PMSF, pH 8. Cells were broken in a cell disrupter. After centrifugation of cell lysates (12,000g, 15 min, 4°C), hPL was found in insoluble fraction (inclusion bodies).
- 10 The inclusion bodies recovered in the pellet were washed 2 times in 250 ml of  $T_{50}E_{0.5}$ , 0.1 mM PMSF, pH 8. Inclusions bodies were solubilized in 20 mM ethanolamine, pH 9 containing 8 M of deionized urea, 1%  $\beta$ -ME, 0.5 mM PMSF at a concentration of 100  $\mu$ g of protein per ml of buffer. The 15 protein solution was heated at 55°C for 5 min and then incubated overnight at room temperature. The renaturation was performed by a continued dialysis (72 hours) against 500 volumes of 20 mM ethanolamine, pH 9.
- 14 The renatured protein was loaded on a HitrapQ anion exchange column. Chromatography was performed in 20 mM ethanolamine, pH 9 and hPL was eluted within a gradient of 0 to 1 M NaCl. Fractions containing hPL were pooled, concentrated to 2 mg/ml and loaded on a Sephadex G100 column (50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5).
- 20 Fractions containing purified hPL were pooled, dialyzed against 20 mM NH<sub>4</sub>HCO<sub>3</sub>, PH 7.5 and lyophilized. Proteins were purified to 95% homogeneity.

Example 11. Purification of the 16K N-terminal Fragment of Human Placental Lactogen

30 This example describes the procedure for preparation of the 16K N-terminal fragment of human placental lactogen (16K hPL (Met<sup>-1</sup>Arg<sup>134</sup>)) (SEQ ID NO:18).

35 For production of 16K hPL, Cys 53 (TGC) was mutated to Ser (TCT) and Arg 134 (CGC) was mutated to Pro (CCC) in order to introduce a thrombin specific cleavage site (SEQ ID NO:14). The mutated cDNA was reinserted into the pT7L expression vector and called pT7L-16K hPL.

An *E. coli* BL21(DE3) culture (100 ml) carrying the plasmid pT7L-16K hPL was grown overnight at 37°C in LB medium-ampicillin 100 µg/ml. Twenty ml of this culture was used to inoculate 1 liter of LB medium-ampicillin 100 µg/ml.

5 When the O.D. (600 nm) reached at 0.9, 1 mM isopropyl β-D thiogalactopyranoside (IPTG) was added to the culture. The induced culture was grown for an additional 4 hour period. Cells were collected by centrifugation at 5000 g for 15 min (4°C) and resuspended in 50 mM Tris-HCl, 0.5 mM EDTA, ( $T_{50}E_{0.5}$ ), 10 0.1 mM PMSF, pH 3. Cells were broken in a cell disrupter. After centrifugation of cell lysates (12,000g, 15 min, 4°C), mutated hPL was found in the insoluble fraction (inclusion bodies).

15 The inclusion bodies recovered in the pellet were washed 2 times in 250 ml of  $T_{50}E_{0.5}$ , 0.1 mM PMSF, pH 8 and stored at -20°C. Inclusions bodies were solubilized in 20 mM ethanalamine, pH 9 containing 8 M of deionized urea, 1% β-ME, 0.5 mM PMSF at a concentration of 100 µg of protein per ml of buffer. The protein solution was heated at 55°C for 5 min and 20 then incubated overnight at room temperature (RT). The renaturation was performed by a continued dialysis (72 hours) against 500 volumes of 20 mM ethanalamine, pH 9. The protein was then concentrated to 2 mg/ml and cleaved with thrombin overnight (0.3%, 25°C).

25 The cleaved proteins were incubated for one hour with 1% β-ME before loading on a Sephadex G100 column (50 mM  $NH_4HCO_3$ , pH 7.5, 100 mM NaCl, 5 mM β-ME). Fractions containing 16K hPL were pooled, dialyzed against 50 mM Tris-HCl, pH 8, 20%  $NH_2SO_4$  and loaded on a phenyl sepharose 6 fast flow 30 hydrophobic column. Chromatography was performed in 50 mM Tris-HCl, pH 8 and 16K hPL was eluted within a gradient 20 to 0% of  $NH_2SO_4$ . Fractions containing purified 16K hPL were pooled, dialyzed against 20 mM  $NH_4HCO_3$ , pH 7.5 and lyophilized. Proteins were purified to 95% homogeneity.

35 The molecular mass of the 16K hPL calculated from its amino acid sequence was determined to be 15.6 kD.

Example 12. Bovine Brain Capillary Endothelial Cell

Proliferation Assay

This example describes bovine brain capillary endothelial cell proliferation assay used for testing in vitro angiogenic activity of the peptides of the invention.

5 On day 1,  $10^4$  BBCE cells prepared as described herein were plated onto 24 well plates (Nunc) in 0.25 ml of low glucose Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal calf serum (Gibco), human bFGF (1 ng/ml, Promega) and concentrations of purified peptides from 0.1, 10 0.2, 0.5, 1, 2, 5, 10, 50 or 100 nM.

Wells containing 0.25 ml of medium well serum without bFGF were included as controls for basal growth. On day 3, bFGF (1 ng/ml) and purified peptides (0.1, 0.2, 0.5, 1, 2, 5, 10, 50 or 100 nM) were added once again to the dishes.

15 15 On day 4, cells were incubated with 500,000 cpm of ( $^3$ H)-thymidine with 0.6  $\mu$ Ci ( $^3$ H)thymidine (20.1 Ci/mmol; New England Nuclear), for 4 hours, washed in 5% trichloroacetic acid, solubilized in NaOH, and counted to method described in *Endocrinology*, 129:896 (1991). Each point represents the means of triplicate wells. The experiments were repeated at least three times, with similar results. Data seen in Figures 1 and 2 are represented as the percentage of bFGF-stimulation. Zero% is the level obtained for basal growth, whereas 100% is obtained when cell proliferation is induced by bFGF.

25 Example 13. Preparation of Bovine Brain Capillary Endothelial Cells

This example illustrates preparation of bovine brain capillary endothelial cells for study of inhibitory effect of human 16K N-terminal fragments of lactogenic hormones.

30 30 Bovine brain capillary endothelial cells (BBCE) were isolated according to *J. Cell. Physiol.*, 127:121 (1986), and grown in low glucose Dulbecco's modified Eagle's medium containing 10 wt% calf serum, 2 mM glutamine, and 100 U/ml penicillin/streptomycin and 2.5  $\mu$ g/ml fungizone.

35 35 Basic fibroblast growth factor (bFGF) was purified from bovine brain according to *P.N.A.S. (USA)* 81: 6963 (1984), and 1 ng/ml was then added to the BBCE cells every other day.

Confluent cell cultures between passages 3-8 were used for the assays.

Example 14. In Gel Mitogen Activated Protein Kinase Assay

5 This example describes in gel mitogen activated protein kinase MAPK assay.

The in gel MAPK assay was carried out essentially as previously described in Proc. Natl. Acad. Sci. U.S.A., 92:6374 (1995).

10 Approximately 20 µg protein from BBCE cell lysates were electrophoresed through a 12.5% SDS-polyacrylamide gel containing 0.5 mg/ml of myelin basic protein (MBP) co-polymerized in the running gel. Following electrophoresis, the gel was washed twice each in buffer A (50 ml Tris pH 8, and 5 mM β-mercaptoethanol) containing 20% isopropyl alcohol, and denatured in buffer A containing 6 M guanidine HCl. After the guanidine HCl wash, the proteins in the gel were allowed to renature at 4°C by extensive washing in buffer A containing 0.04% Tween 40. Renatured MBP kinase activity was detected by 15 incubating the gel for 60 min at room temperature in a reaction buffer containing 40 mM HEPES (pH 7.4) 2 mM DTT, 15 mM MgCl<sub>2</sub>, 300 µM sodium orthovanadate, 100 mM EGTA, 25 µM ATP and 100 µCi of ( $\gamma$ -<sup>32</sup>P)ATP. Unincorporated radioactivity was removed by extensive washing in 5% trichloroacetic acid 20 containing 1% tetrasodium pyrophosphate PPi. The gel was dried and exposed to X-ray film for 24 hours.

25 Example 15. Phosphotyrosine Western for Mitogen Activated Protein Kinase

This example describes Western blot for detection of 30 mitogen activated protein kinase (MAPK) used for determination of specificity of the receptors bind peptides of the invention.

Cellular proteins were resolved by SDS-PAGE (8%, 12% 35 12.5%) and transferred to nitrocellulose membranes (Schleicher & Schuell). Western blots were probed with the following antibodies: an anti-phosphotyrosine mouse monoclonal antibody

(UBI, USA) (4G10, 1:2,000 dilution) and an anti-MAPK polyclonal antiserum that recognizes both p42 and p44 MAPK (erk 1-CT, 1:10,000 dilution). Western blots were incubated with the appropriate antibody and then washed in Tris-buffered saline containing 0.5% Nonidet NP-40 and 0.1% Tween 20.

5 Antigen-antibody complexes were detected with horseradish peroxidase-coupled secondary antibodies using the Enhanced Chemiluminescence system (ECL, Amersham). The blots were exposed to Reflection NEF films (NEN). Western blots were  
10 stripped for reprobing with other primary antibodies by incubation for 30 min at 22°C in a buffer containing 0.2 M glycine (pH 2.5) followed by two washes in PBS.

Example 16. Western blot Analysis for Bovine Plasminogen Activator Inhibitor-1

15 This example describes the assay used for detection of inhibition of bovine plasminogen activator inhibitor-1 by peptides of the invention.

20 Cell homogenates or conditioned media from BBCE cells were resolved by SDS-PAGE (4-10%) and transferred to nitrocellulose membrane by semi-dry transfer apparatus. The transfer blots were stained with Ponceau Red for 1 min to visualize the even transfer of the proteins. The blots were blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 for 1 hour and incubated with anti-bovine PAI-1 mouse  
25 monoclonal antibody at a 1:2,000 dilution for 2 hours obtained from (Gibco Gaithersburg, MD).

30 The antigen-antibody complexes were detected with horseradish peroxidase conjugated secondary antibody and the Enhanced Chemiluminescence system. The blots were exposed to reflection EF films (NEN) to visualize the bands.

Example 17. Chick Chorioallantoic Membrane Assay

This example illustrates chick chorioallantoic membrane assay used for testing of *in vivo* inhibitory activity of peptides of the invention.

35 Chick embryos were placed in a Petri dish on day 3 of their development and cultured in a humidified incubator in

an atmosphere of oxygen containing 2.5% CO<sub>2</sub> as described in Nature, 297:307 (1982). On day six, 5 mm disk of methylcellulose (0.5%, Sigma M0512) containing 20 µg of peptides of the invention and 2 µg bovine serum albumin (BSA) 5 were laid on the advancing edge of the chick CAM as previously described in Nature, 297:307 (1982). After 48-h exposure, white India ink was injected into the chorioallantoic sac for photographic purpose.

All publications and patent applications cited in 10 this specification are herein incorporated by reference in their entirety for all purposes as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

As will be apparent to those skilled in the art to 15 which the invention pertains, the present invention may be embodied in forms other than those specifically disclosed above, without departing from the spirit or essential characteristics of the invention. The particular embodiments of the invention described above, are, therefore to be 20 considered as illustrative and not restrictive. The scope of the present invention is as set forth in the appended claims rather than being limited to the examples contained in the foregoing description.

WHAT IS CLAIMED IS:

- 1               1. An anti-angiogenic peptide substantially  
2 identical to about 10 to about 150 consecutive amino acids  
3 selected from the N-terminal end of human placental lactogen,  
4 human growth hormone, growth hormone variant hGH-V, or human  
5 prolactin, wherein the peptide
  - 6               (i) inhibits capillary endothelial cell  
7 proliferation and organization;
  - 8               (ii) inhibits angiogenesis in chick chorioallantoic  
9 membrane; and
  - 10              (iii) and binds to at least one specific receptor  
11 which does not bind an intact full length growth hormone,  
12 placental lactogen, prolactin, or growth hormone variant hGH-  
13 V.
- 1               2. The peptide of claim 1, wherein the peptide is  
2 generated by enzymatic cleavage of growth hormone, placental  
3 lactogen, prolactin, or growth hormone variant hGH-V.
- 1               3. The peptide of claim 1 having the amino acid  
2 sequence of SEQ ID NO:18.
- 1               4. The peptide of claim 1 having the amino acid  
2 sequence of SEQ ID NO:24.
- 1               5. The peptide of claim 1 having the amino acid  
2 sequence of SEQ ID NO:30.
- 1               6. An isolated nucleic acid encoding the peptide  
2 of claim 1.
- 3               7. The nucleic acid of claim 6, wherein the  
4 nucleic acid is DNA.
- 5               8. The nucleic acid of claim 1, wherein the  
6 nucleic acid is RNA.

1           9. The nucleic acid of claim 7, wherein the  
2 nucleic acid comprises a cDNA sequence.

1           10. The nucleic acid of claim 7, wherein the  
2 nucleic acid comprises the sequence of SEQ ID NO:14.

1           11. The nucleic acid of claim 7, wherein the  
2 nucleic acid comprises the sequence of SEQ ID NO:20.

1           12. The nucleic acid of claim 7, wherein the  
2 nucleic acid comprises the sequence of SEQ ID NO:26.

1           13. The nucleic acid of claim 7, wherein the  
2 nucleic acid comprises the sequence of SEQ ID NO:19.

1           14. The nucleic acid of claim 7, wherein the  
2 nucleic acid comprises the sequence of SEQ ID NO:13.

1           15. The nucleic acid of claim 7, wherein the  
2 nucleic acid comprises the sequence of SEQ ID NO:25.

1           16. The nucleic acid sequence of claim 1, wherein  
2 the nucleic acid sequence comprises a vector.

1           17. The nucleic acid sequence of claim 16, wherein  
2 the vector is an expression vector.

1           18. A host cell comprising the nucleic acid of  
2 claim 7.

1           19. A method of producing the peptide of claim 1,  
2 comprising expressing the nucleic acid in the host cell of  
3 claim 18, and recovering the peptide.

1           20. The method of claim 19, wherein a peptide  
2 product of the expressed nucleic acid is recovered from the  
3 host cell and is enzymatically cleaved to generate the peptide  
4 of claim 1.

1           21. A method of treating an angiogenic disease in a  
2 subject, the method comprising administering to a subject in  
3 need of such treatment an angiogenesis inhibitory effective  
4 amount of a peptide of claim 1.

1           22. The method of claim 21 wherein the peptide has  
2 the amino acid sequence of SEQ ID NO:18, SEQ ID NO:24 or SEQ  
3 ID NO:30.

1           23. A method of inhibiting tumor formation or  
2 growth in a patient, the method comprising administering to  
3 the patient an angiogenesis inhibitory effective amount of the  
4 peptide of claim 1.

1           24. The method of claim 23 wherein the peptide has  
2 amino acid sequence SEQ ID NO:18, SEQ ID NO:24 or SEQ ID  
3 NO:30.

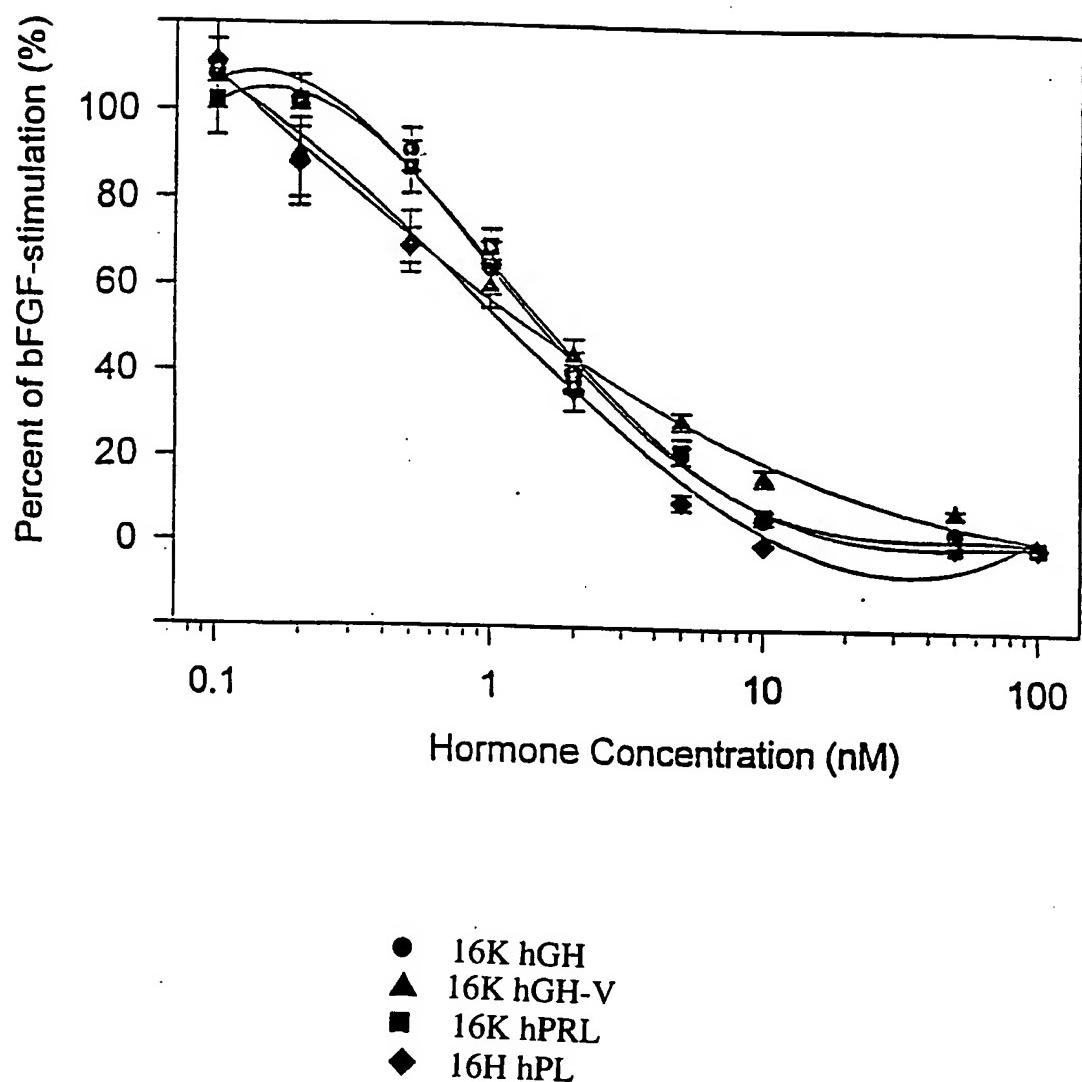
1           25. A method for diagnosing a probable abnormality  
2 of placental vascularization during pregnancy comprising  
3 assaying a level of at least one of endogenous N-terminal  
4 fragments of growth hormone, prolactin, growth hormone variant  
5 hGH-V, and placental lactogen in a tissue sample from a  
6 patient; and

7           comparing the level of the at least one of  
8 endogenous N-terminal fragments to an average level of the at  
9 least one of endogenous N-terminal fragments in a normal  
10 patient population;

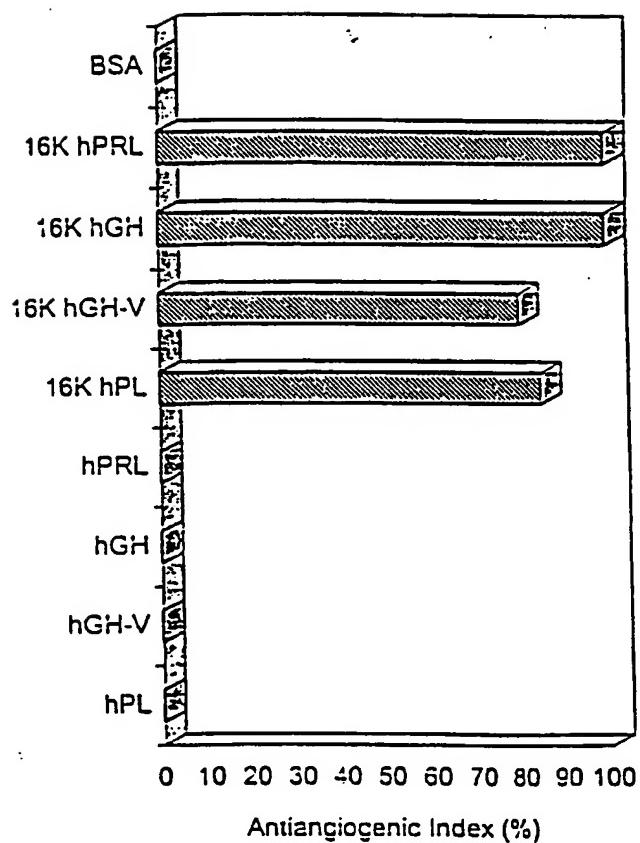
11           wherein a level of the at least one of endogenous N-  
12 terminal fragments higher than the average level is a probable  
13 abnormality of placental vascularization during pregnancy.

1           26. A method of modulating vascularization of a  
2 patient's placenta, the method comprising administering to the  
3 patient an angiogenesis inhibitory effective amount of the  
4 peptide of claim 1.

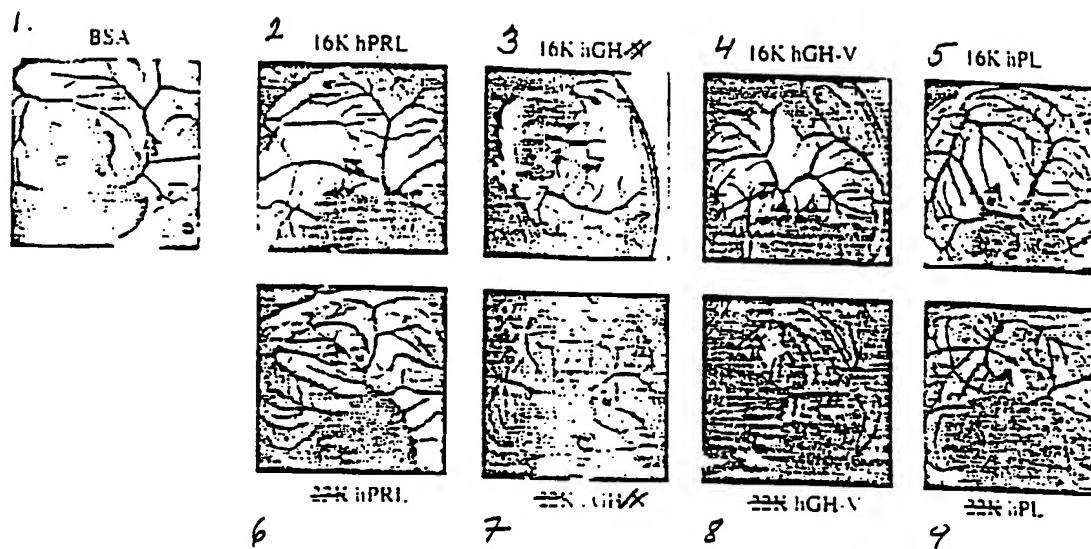
FIG. 1

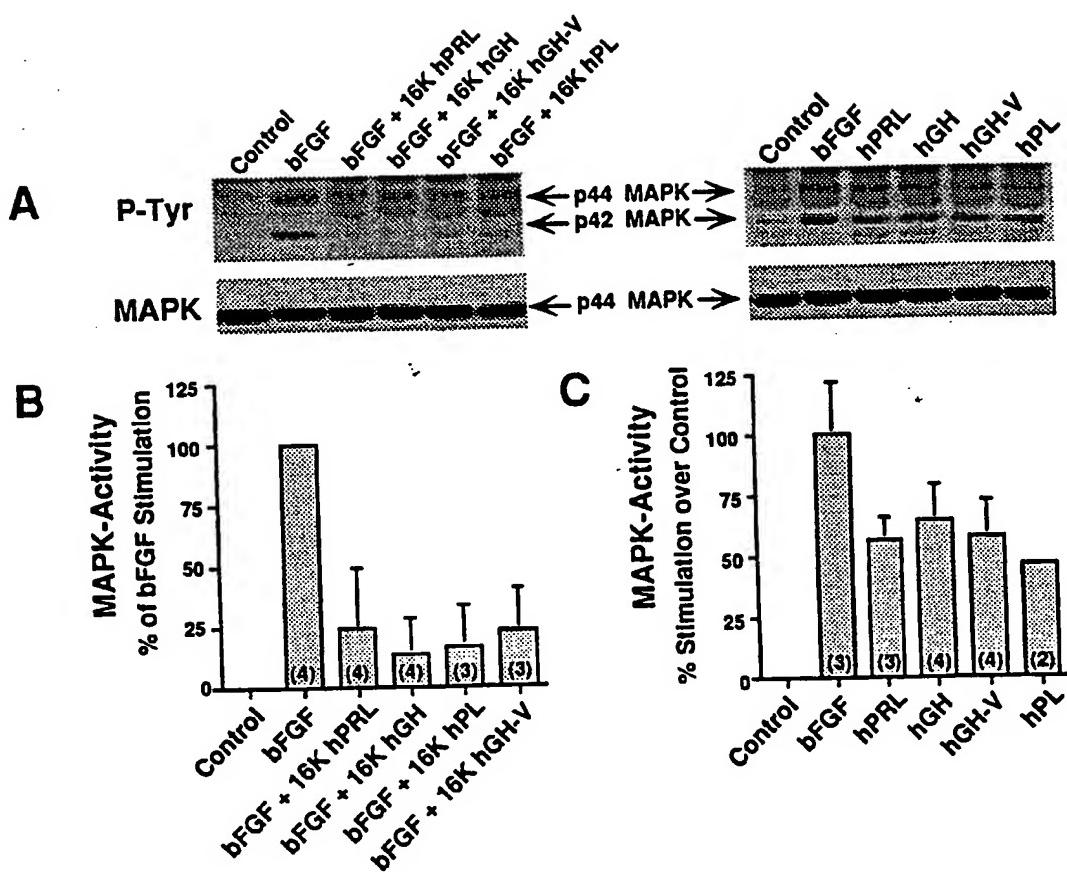


F16.2

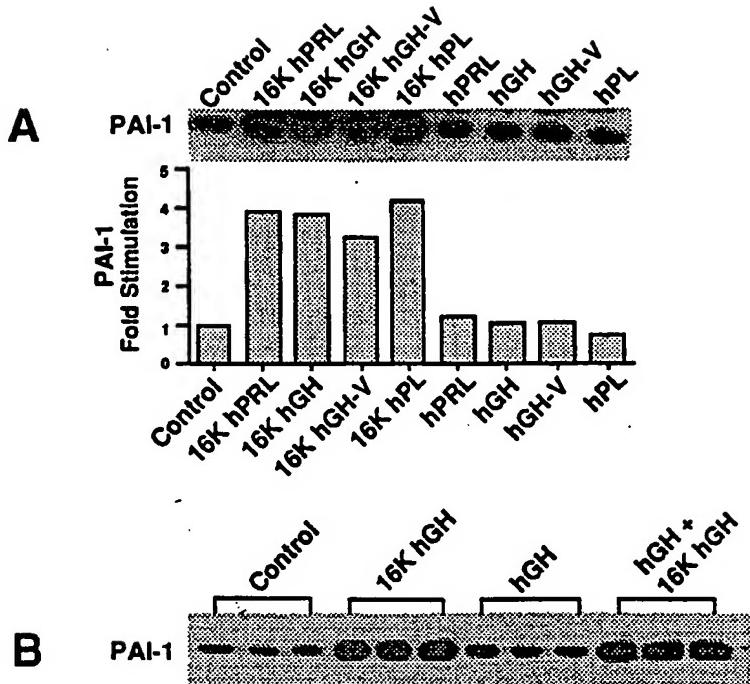


E163



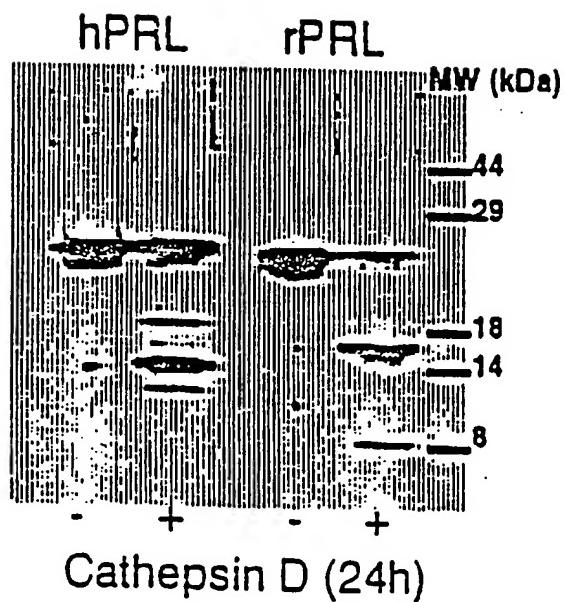


New figure 4



New figure 5

FIG. 6



F16.7

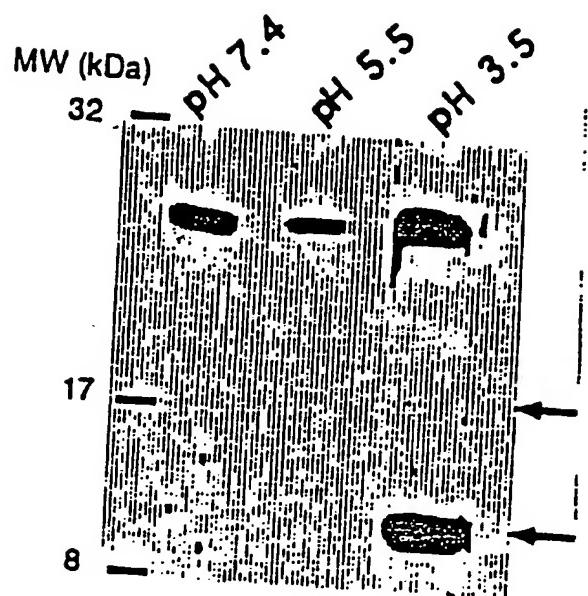


FIG. 8A

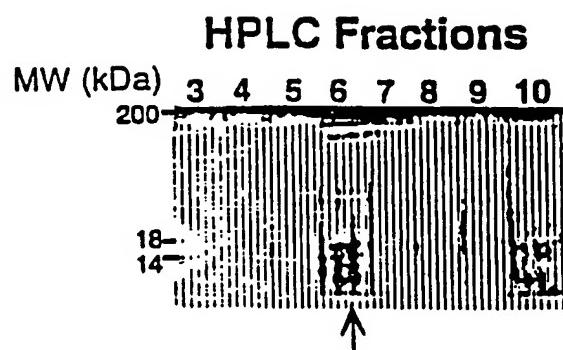
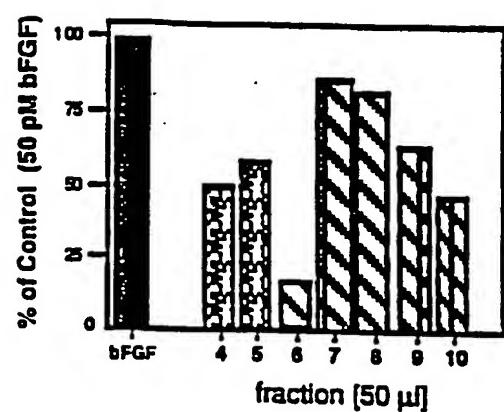


FIG. 8B



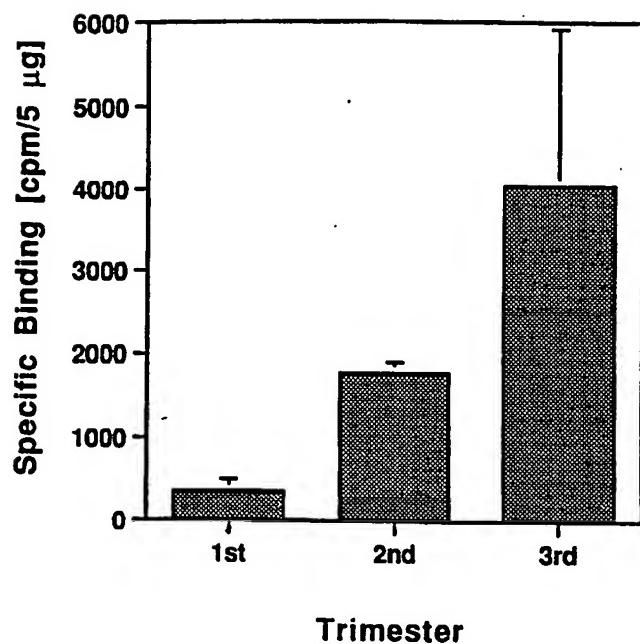


Figure 9

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/09691

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 38/00, 31/04, 31/10, 31/16; C07K 14/00  
US CL : 514/02; 530/300

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/02; 530/300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, APS ON LINE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	RUSSELL, J. et al. Recombinant Hormones from Fragments of Human Growth Hormone and Human Placental Lactogen. Journal of Biological Chemistry. 10 January 1981, Vol. 256, No. 1, pages 296-300, see entire document.	1,2
X -- Y	Schneider, A. et al. Conformation of Biologically Active Derivatives of Human Placental Lactogen. Journal of Biological Chemistry. 10 January 1981, Vol. 256, No. 1, pages 301-303, see entire document.	1,2

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
*O* document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

04 AUGUST 1998

Date of mailing of the international search report

21 AUG 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Faxsimile No. (703) 305-3230

Authorized officer

MICHAEL BORIN

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/09691

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
  
  
  
2.  Claims Nos.: 3-5 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
  
The claims refer to SEQ ID numbers of peptides, but no sequence listing was provided.
  
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
  
  
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1,2, first group of species

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/09691

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**  
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

- I. Claims 1,2 drawn to peptides.
- II. Claims 6-9,16-18, drawn to nucleic acids, transformed host cells.
- III. Claims 19-20, drawn to method of making of peptides of Group I.
- IV. Claims 21, method of treating angiogenic disease.
- V. Claim 23, drawn to method of inhibiting tumor formation.
- VI. Claim 25, drawn to method of diagnosing placental vascularization abnormality.
- VII. Claim 26, drawn to method for modulating vascularization of placenta.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species, as applied to Group I, are as follows:

- peptides having homology to human placental lactogen;
- peptides having homology to human growth hormone;
- peptides having homology to growth hormone variant hGH-V;
- peptides having homology to human prolactin;

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is the technical feature that links Groups I to V. Group I is not the contribution over the prior art because it is suggested by references teaching peptides substantially identical to, e.g., human placental lactogen, and having a length of 10-150 residues. For example, Schneider et al. ( J. Biol. Chem. (1981), 256(1), pages 296-300 and 301-303) teach various fragments of human growth hormone and human placental lactogen, such as:

VQTVPLSRLF DHAMLQAHRA HQLAIDTYQE FEETYIPKDDQ KYSFLHDSQT  
SFCFSDSIPT PSNMEETQQK SNLELLRISL LLIESWLEPV RFLRSMFANN  
. LVYDTSDSDD YHLLKDLEEG IQTLMGRLED GSRR;

VQTVPLSRLF DHAMLQAHRA HQLAIDTYQE FEETYIPKDDQ KYSFLHDSQT  
SFCFSDSIPT PSNMEETQQK SNLELLRISL LLIESWLEPV RFLRSMFANN

Therefore, the lack of unity is present because the linking technical feature is not a "special technical feature" as defined by PCT Rule 13.2.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: the species are structurally different as they are derived from proteins having different structure.